

PATENT
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Hans DECKMYN et al.	Art Unit:	1644
Serial No.:	10/049,868	Examiner:	Maher M. Haddad
Filed:	June 4, 2002	Customer No.:	21559
Title:	CELL LINES, LIGANDS AND ANTIBODY FRAGMENTS FOR USE IN PHARMACEUTICAL COMPOSITIONS FOR PREVENTING AND TREATING HAEMOSTASIS DISORDERS		

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APPEAL BRIEF ON APPEAL PURSUANT TO 37 C.F.R. § 41.37

In support of Appellants' Notice of Appeal that was filed in connection with the above-captioned case on December 1, 2005, and with reference to the final Office Action mailed May 27, 2005 and Advisory Actions mailed respectively December 30, 2005 and April 24, 2006, submitted herewith is appellants' Appeal Brief.

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Real Party in Interest

The real party in interest in this case is K.U. Leuven Research & Development, to whom all interest in the present application has been assigned.

Related Appeals and Interferences

There are no pending appeals or interferences related to this case.

Status of Claims

Claims 65-66, 70-75, and 80-83 are pending. Claims 70, 71, 75, and 80-83 would be allowable if submitted in a separate, timely filed amendment. Claims 65 and 66 were rejected under § 103(a) and are addressed in this appeal.

Status of Amendments

Appellants' amendment dated June 9, 2006 cancelling claims 72-73 and 83 has not been entered. Claims Appendix B includes the pending claims (Claims 65-66, 70-71, and 80-82) as reflected upon entry of the June 9, 2006 amendment.

Summary of Claimed Subject Matter

Appellants' invention generally features a pharmaceutical composition which includes a monovalent antibody fragment which binds *in vivo* to human platelet glycoprotein GPIb without incurring thrombocytopenia and a pharmaceutically acceptable carrier (claim 65; WO 01/10911, p. 6, ll. 29-32). In a desirable embodiment, the fragment is a Fab fragment or a single variable domain (claim 66; WO 01/10911, p. 10, ll. 15-32 and p.1, ll. 6). In other desirable embodiments, the variable region of the fragment includes SEQ ID NO: 4 (claim 70; WO 01/10911, Figure 13). In still another desirable embodiment, the monovalent antibody fragment is obtained from a monoclonal antibody produced by the cell line deposited with the Belgian Coordinated Collections of Microorganisms, under accession number LMBP 5108CB (claim 7; WO 01/10911, p. 10, ll. 17-20).

Grounds of Rejection to be Reviewed on Appeal

Appellants submit that the Office erred, in its final Office Action mailed May 27, 2005 and Advisory Actions mailed respectively December 30, 2005 and April 24, 2006, in finally rejecting claims 65 and 66 under 35 U.S.C. § 103(a) as being unpatentable over Ward et al. ("Epitope and functional characterization of the CD42 (gpIb/IX) mAb panel." *Platelet Antigens*: 1336-1337, 1995) in view of Owens et al. ("The Genetic Engineering of Monoclonal Antibodies." *Journal of Immunological Methods* 168:149-165 (1994)) and

Tsurumizu, U.S. Patent No. 4,731,245 (the “245 patent”) titled “Vaccine, Antigen, and Antibody for Treating Microorganisms of the MCLS-Type Streptococcus Sanguis Kawasaki Disease.”

Argument

Rejections under 35 U.S.C. § 103(a)

As discussed above, claims 65 and 66 were finally rejected under 35 U.S.C. § 103(a) as being unpatentable over Ward et al. (1995) in view of Owens et al. (1994) and U.S. Patent No. 4,731,245 (hereafter “the ‘245 patent”). Appellants address each of these rejections as follows.

The Claimed Invention

Appellants’ application, in general, includes claims directed to a pharmaceutical composition. Claims 65 and 66 read as follows:

65. A pharmaceutical composition comprising a monovalent antibody fragment which binds *in vivo* to human platelet glycoprotein GPIb without incurring thrombocytopenia and a pharmaceutically acceptable carrier.
66. The pharmaceutical composition according to claim 65, wherein said fragment is a Fab fragment or a single variable domain.

The Cited References

Ward

In characterizing the Ward reference, the Office states:

Ward *et al* teach 17 monoclonal antibodies that bind GPIIb α epitope of the platelet surface glycoprotein (see table 1 in particular). Ward et al teach that eight antibodies mapped to the N-terminal fragments of gplba, and these were tested for their ability to block binding of 125I-labelled von Willebrand factor to washed platelets in the presence of ristocetin or botrocetin. Ward et al teach that mAb P0 14 (epitope 1-282), P024 (epitope 1-282), P073 (epitope 1-282). P074 (epitope 1-282) and P077 (epitope 1-282 completely inhibited vWF binding with [either] modulator (see page 1337, 1st col., 3rd paragraph and table 1 in particular). Finally, Ward et al teach that the inhibitory functions of the CD42b antibodies with their epitopes on gplba may provide valuable insights into mechanisms of vWF function both in vitro and in vivo (pg 1337, last paragraph in particular).

Owens

In connection with Owens, the Office asserted:

[T]he modification of murine antibodies such as single chain antibody, a Fab fragment or a humanized antibody using monoclonal antibody technology. Owens *et al* further teach humanized antibodies [used] in therapy of human diseases or disorders, since human or humanized antibodies are much less likely to induce an immune response. Also, antibody fragments are the reagents of choice for some clinical applications (see the entire document).

The '245 patent

In addition, the Office, in connection with the '245 patent, states:

The '245 patent teaches a composition comprising the antibody to the PLS antigen, as the active ingredient in association with a pharmaceutically acceptable carrier or excipient. The composition may preferably take the forms suitable for oral administration. Advantageously, the composition may be formulated in dosage unit form. The amount of the active ingredient contained in each dosage unit may be adjusted so as to enable the administration of the

antibody at a daily dose (see col., 7 line 63 through col., 8 line 3 in particular).

Based on these references, the Office concluded that “the invention taken as a whole is *prima facie* obvious over the prior art.” In reaching this conclusion, the Office explained:

It would have been obvious to one of ordinary skill in the art at the time the invention was made to produce the monoclonal antibody taught by Ward et al as Fab as taught by the Owens *et al* and place the resultant Fab fragment which binds to platelet glycoprotein GPIIb polypeptide taught by the Ward et al reference in a composition taught by the ‘245 patent.

One of ordinary skill in the art at the time the invention was made would have been motivated to do so because eight antibodies mapped to the N-terminal fragments of gplIb, and these were tested for their ability to block binding of ¹²⁵I-labelled von Willebrand factor to washed platelets in the presence of ristocetin or botrocetin and because it would further lead to insights into mechanisms of vWF function both in vitro and in vivo. Given that the antibody fragments are the reagents of choice for some clinical applications one ordinary skill in the art at the time the invention was made would be motivated to include such fragments in a composition because the composition can be formulated in dosage unit form. The amount of the active ingredient contained in each dosage unit may be adjusted so as to enable the administration of the antibody at a daily dose as taught by ‘245 patent.

This rejection should be withdrawn, as it is applied to claim 65 and claim 66, because the asserted motivation for combining these references is unsupported by the references themselves. No *prima facie* case for obviousness exists in this case.

Analysis

In determining whether an invention is obvious, the Office must determine if “the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains.”

35 U.S.C. § 103. The factual inquiries underlying obviousness include (1) the scope and content of the prior art, (2) the differences between the prior art and the claims at issue, (3) the level of ordinary skill in the art at the time the invention was made, and (4) any objective evidence of nonobviousness. *Graham v. John Deere Co.*, 383 U.S. 1, 17-18, 15 L. Ed. 2d 545, 86 S. Ct. 684 (1966). “The consistent criterion for determination of obviousness is whether the prior art would have suggested to one of ordinary skill in the art that this process should be carried out and would have a reasonable likelihood of success, viewed in the light of the prior art.” *In re Dow Chem. Co.*, 837 F.2d 469, 473 (Fed. Cir. 1988). Obviousness requires one of ordinary skill in the art have a reasonable expectation of success as to the invention--“obvious to try” and “absolute predictability” are incorrect standards. *In re O'Farrell*, 853 F.2d 894, 903 (Fed. Cir. 1988).

The Federal Circuit has further stated:

“[V]irtually all [inventions] are combinations of old elements.”
Therefore an examiner may often find every element of a claimed invention in the prior art. If identification of each claimed element in the prior art were sufficient to negate patentability, very few patents would ever issue.

In re Rouffet, 149 F.3d 1350, 1357-58, 47 USPQ2d 1453, 1457 (Fed. Cir.

1998) (internal citations omitted).

“Both the suggestion and the expectation of success must be founded in the prior art, not in the applicant’s disclosure.” *In re Dow Chem. Co.*, 837 F.2d 469, 473, 5 U.S.P.Q.2d 1529, 1531 (Fed. Cir. 1988). As the Federal Circuit has observed (emphasis added):

A critical step in analyzing the patentability of claims pursuant to section 103(a) is casting the mind back to the time of invention, to consider the thinking of one of ordinary skill in the art, guided only by the prior art references and the then-accepted wisdom in the field. . . . Most if not all inventions arise from a combination of old elements. . . . Thus, every element of a claimed invention may often be found in the prior art. . . . However, identification in the prior art of each individual part claimed is insufficient to defeat patentability of the whole claimed invention. . . . Rather, to establish obviousness based on a combination of the elements disclosed in the prior art, there must be some motivation, suggestion or teaching of the desirability of making the specific combination that was made by the applicant.

In re Kotzab, 217 F.3d 1365, 1369-70, 55 U.S.P.Q.2d 1313, 1316 (Fed. Cir. 2000) (citations omitted) (emphasis added).

Moreover, the evidence of a suggestion, teaching, or motivation to combine “must be clear and particular” (emphasis added).” *Dembiczak*, 175 F.3d at 999, 50 U.S.P.Q.2d at 1617. “Defining the problem in terms of its solution reveals improper hindsight in the selection of the prior art relevant to obviousness.” *Monarch Knitting Mach. Corp. v. Sulzer Morat GMBH*, 139 F.3d 877, 881, 45 U.S.P.Q.2d 1977, 1981 (Fed. Cir. 1998). Thus, even if the Examiner identifies every element of a claimed invention in the prior art,

this alone is insufficient to negate patentability. Otherwise, “rejecting patents solely by finding prior art corollaries for the claimed elements would permit an examiner to use the claimed invention as a blueprint for piecing together elements in the prior art to defeat the patentability of the claimed invention.” *Rouffet*, 149 F.3d 1350, 1357, 47 U.S.P.Q.2d 1453, 1457 (Fed. Cir. 1998). To avoid hindsight based on the invention to defeat patentability of the invention, the Federal Circuit requires an Examiner to show a motivation to combine the references that create the case of obviousness. *Id.* That is, “the examiner must show reasons that the skilled artisan, confronted with the same problems as the inventor and with no knowledge of the claimed invention, would select the elements from the cited prior art references for combination in the manner claimed.” *Id.* at 1357, 1458 (emphasis added). The Supreme Court recognized the hindsight problem in *Graham* and proposed that “legal inferences” resulting from “secondary considerations” might help to overcome it. 383 U.S. at 36 (“[Secondary considerations] may also serve to guard against slipping into use of hindsight, and to resist the temptation to read into the prior art the teachings of the invention in issue.” (internal quotations omitted)).

As explained below, the Office has failed to show a *prima facie* case of obviousness and the rejections of claim 65 and claim 66 should therefore be withdrawn.

Ward, Owens, and the '245 Patent Fail to Motivate, Teach, or Suggest the Invention of Claim 65 or Claim 66

As motivation for combining the cited references, the Office states that “because eight antibodies mapped to the N-terminal fragments of gp1ba and because it would lead to insights into the mechanisms of vWF function.” There is, however, nothing in the references of record that provides any basis for selecting a monovalent antibody fragment that binds *in vivo* to GP1b or to use such a fragment in a pharmaceutical composition, and the Office’s analysis amounts to merely an invitation to experiment.

First, the Office’s statement that “[g]iven that the antibody fragments are reagents of choice for some clinical applications one [of skill in the art] would be motivated to include such fragments in a composition,” is plainly predicated on an improper “obvious to try” standard. It is insufficient that one skilled in the art might find it “obvious to try” combining the Ward, Owens, and ‘245 patent references. As the Federal Circuit has held, an obvious to try situation does not render a claim “obvious” within the meaning of section 103. (“An invention is obvious to try rather than obvious within the meaning of § 103 ““where the prior art [gives] either no indication of which parameters [are] critical or no direction as to which of many possible choices is likely to be successful.”” *Merck & Co., Inc. v. Biocraft Labs., Inc.*, 874 F.2d 804, 807 (Fed. Cir. 1989) (quoting *In re O’Farrell*, 853 F.2d 894, 903 (Fed. Cir. 1988)).)

It is undisputed that neither Ward, Owens, nor the ‘245 patent contains an express suggestion of either “a monovalent antibody fragment which binds *in vivo* to ... GP1b,”

much less a “pharmaceutical composition comprising a monovalent antibody fragment which binds *in vivo* to ... GP1b.” Indeed, nothing in the cited references would have suggested to a person of ordinary skill in the art that a pharmaceutical composition that includes a monovalent antibody would function *in vivo* without incurring thrombocytopenia as claimed. Moreover, the Office, apart from pointing out that Ward teaches 17 monoclonal antibodies that bind GP1b α epitope, provides no scientific evidence or reasoning that there would have been a “reasonable expectation of success” in creating a pharmaceutical composition as claimed.

The Examiner has referred to Ward as disclosing antibodies that bind GP1b α epitope. On the contrary, Ward relates to *intact* monoclonal antibodies which are shown to inhibit vWF binding and ristocetin and botrocetin-induced platelet aggregation *in vitro*. Ward et al. speculates that “*further studies comparing inhibitory functions may provide valuable insights into the mechanisms of vWF function in vitro and in vivo*” (emphasis added), but provides no *in vivo* data. It is clear that Ward was only *beginning to investigate* GP1b antibodies. Indeed, Ward’s antibodies were developed to perform epitope-mapping *in vitro*. Although these data may help to explain how proteins interact *in vivo*, they are not necessarily useful as pharmaceutical compositions. This reference provides no scientific or logical predicate for rendering Appellants’ claims obvious.

Next, appellants point out that Ward carried out *in vitro* experiments, which, as is well known, frequently fail to be predictive of *in vivo* results. Claim 65 requires that the

monovalent antibody fragment bind *in vivo* ... without incurring thrombocytopenia. Ward fails to teach a single monovalent antibody to GP1b. Moreover, nothing in the Ward *in vitro* experiments takes into account whether thrombocytopenia is prevented *in vivo*.

Indeed, Appellants note the invention of claim 65 relates to the use of monovalent GP1b antibody fragments as pharmaceutical compositions because it is based on Appellants' discovery that monovalent antibody fragments are capable of inhibiting GP1b *in vivo* platelet aggregation (which is required for an anti-thrombotic effect) without causing thrombocytopenia. Furthermore, as is discussed below, the skilled person at the time of filing of the present application was aware of the thrombocytopenia induced by antibodies against GP1b based on the publications describing *in vivo* experiments in animal models, and therefore would not be motivated to use such antibodies as pharmaceutical compositions. Appellants further note that there is no indication in the art as to what caused this thrombocytopenic side-effect. Accordingly, Appellants submit that it was unobvious for the skilled person to develop a pharmaceutical composition that includes a monovalent antibody fragment that binds *in vivo* to GP1b at the time of filing.

In addition, it is inappropriate for the Office to view Owens, Ward, and the '245 patent in hindsight and with the success of Appellants' invention in mind. Instead, what is required is that the Office must "cast its mind back to the time of the invention," as required by *In re Dow* and be guided "*only* by the prior art references and the then-accepted wisdom in the field." *In re Dow*, 837 F.2d at 473 (emphasis added).

If this standard is applied, it is clear that a skilled worker reading Ward would not be motivated to look to Owens or the '254 patent or both. Ward never indicates that one of the 17 monoclonal antibodies that bind GP1b α would be beneficial for therapeutic administration. Ward makes no mention of antibody fragments, and further never teaches or suggests that monovalent fragments of the anti-GP1b α antibodies be produced. Ward, as mentioned above, is also uncertain as to the significance of their own findings, concluding “[f]urther studies ... may provide valuable insights into the mechanisms of vWF function *in vitro* and *in vivo*.” What are the valuable insights into the mechanisms of vWF function? At best, Ward advocates additional research. Ward provides no “clear and particular” direction for using any of the eight disclosed antibodies as therapeutics.

With respect to Ward, the Office also asserts that “Ward has done nothing different [than] the [Appellants’] specification with respect to *in vivo* data.” Appellants disagree. Unlike Ward, which provides no opinion on *in vivo* function of the anti-GP1b antibodies, Appellants teach, for example, *in vivo* baboon studies (Examples 7-9).

Owens and the '245 patent never mention antibodies that bind human glycoprotein GP1b much less suggest that the claimed antibodies would be beneficial therapeutics.

Turning now to Owens, this reference describes the modification and production of antibodies or fragments by genetic engineering. Different types of antibodies and fragments are discussed. Generally, problems encountered with natural antibodies are described. In the context of the construction of Fv and single chain Fv fragments, the

advantage of Fvs over Fab antibodies is presented. Owens does not specifically describe the advantages of monovalent antibody fragments (Fabs or Fvs) over complete antibodies or F(ab)2 fragments. Given this teaching, Owens plainly does not suggest Appellants' claimed invention. Accordingly, Owens cannot teach or suggest what they themselves did not know or recognize. The Ward and Owens references are unavailing, and, in combination, cannot support the present obviousness rejection.

The '245 patent is cited for teaching a composition that includes antibody as an active ingredient. It provides no information regarding Appellants' claimed invention either alone or in combination with Ward or Owens. Both references are unavailing because each merely provides methods known at the time the application was filed. None, alone or in combination with Ward, provides a single insight into the *in vivo* therapeutic activity of the claimed pharmaceutical compositions.

The Ward, Owens, and '245 patent prior art combination is not the type of "clear and particular" motivation required by the Federal Circuit. *In re Dembiczak*, 175 F.3d at 999. Without motivation for the combination of references, no *prima facie* case of obviousness can exist, and the § 103 rejection on this basis alone must be withdrawn.

**Cadroy, Bergmeier, and Phillips Teach Away From
the Invention of Claims 65 or 66 or both**

Appellants further note that a number of anti-GP1b antibodies had been described in the art at the time of filing of the present application. Despite their ability to block

ristocetin-induced platelet aggregation *in vivo*, these antibodies would not be considered as suitable pharmaceutical compounds by the skilled person as these antibodies were also found to induce thrombocytopenia when used *in vivo*. As evidence of this assertion, Appellants direct the Examiner's attention to the publication of Cadroy *et al.*, *Blood* 83:3218-3224 (1994) and Bergmeier *et al.*, *Blood* 95:886-893 (2000), which both describe the administration of monoclonal antibodies to GP1b or F(ab)2 fragments thereof *in vivo* in animal models. In contrast to Appellants' claimed invention, both articles report the immediate induction of thrombocytopenia upon administration of the antibody.

The Office, in its final Action, attributed little weight to Appellants' reliance on the Cadroy and Bergmeier references, stating:

Both references used either an intact antibody or a divalent antibody, but not monovalent antibody. Again the resultant antibody fragment of Fab or scFv would not be expected to cause thrombocytopenia.

Bergmeier, makes clear, however, at page 892 (2nd paragraph) that "attempts to block certain epitopes on GP1b with modified antibodies may generally result in thrombocytopenia....[and] *in vivo* blockage of certain epitopes on GP1b may, therefore, not be a promising antithrombotic strategy." (emphasis added.) Bergmeier teaches that antibody binding to GP1b results in thrombocytopenia, regardless of whether the antibody is a F(ab)₂, Fab, or scFv. Indeed, Bergmeier concedes that thrombocytopenia results from the binding between the antibody and its GP1b epitope.

Bergmeier discourages one skilled in the art away from Appellants' monovalent antibody fragment pharmaceutical composition antithrombotic strategy. Bergmeier's teaching would lead a person of ordinary skill, upon reading the reference, in a direction divergent from the path that was taken by Appellants. The skilled worker would not be led to construct additional antibody fragments such as Fabs or scFvs when Bergmeier teaches that antibody binding to GP1b results in thrombocytopenia, much less use such fragments in a pharmaceutical composition. For this reason as well, the obviousness rejection in this case must be withdrawn. See, for example, *In re Haruna*, 249 F.3d 1327, 1335, 58 U.S.P.Q.2d 1517, 1522 (Fed. Cir. 2001) ("A prima facie case of obviousness can be rebutted if the applicant ... can show 'that the art in any material respect taught away' from the claimed invention *A reference may be said to teach away when a person of ordinary skill, upon reading the reference, ... would be led in a direction divergent from the path that was taken by the applicant.*") (emphasis added) (citations omitted).

Indeed, as further evidence that use of antibodies against GP1b as therapeutics was discouraged by workers in the field, Appellants, in their reply to the final office action, further directed the examiner's attention to Phillips et al. (Therapeutic approaches in arterial thrombosis, *Journal of Thrombosis and Haemostasis* 3:1577-1589, 2005) at page 1583, left column, where it is stated that "[m]odulation of the VWF/GPIb α axis has been the subject of many investigations with promising animal experimental results, but severe thrombocytopenia has been associated with the use of antibodies against GPIb α , thus

reducing the general interest of the scientific community for several years.” Given this statement too, it is unreasonable to assume that one skilled in the art, at the time the application was filed, would have been motivated to combine the teachings of Ward, Owens, and the ‘245 patent in the manner suggested by the Office.

The Office’s Obviousness Analysis is Based on Impermissible Hindsight

Finally, the Office has failed to explain, when analyzing the references made of record, what specific understanding would have suggested the combination of references relied on by the Office, especially in view of the Appellants’ results. Instead, the obviousness analysis is limited to a discussion of how the references can be pieced together to yield the claimed invention. As the Federal Circuit stated in *Interconnect Planning Corp. v. Feil*, 774 F.2d 1132, 227 U.S.P.Q. 543 (Fed. Cir. 1985):

It is an error to reconstruct the patentee’s claimed invention from the prior art by using the patentee’s claim as a “blueprint.” When prior art references require selective combination to render obvious a subsequent invention, there must be some reason for the combination other than the hindsight obtained from the invention itself.

To believe that one skilled in the art would be motivated to generate Appellants’ disclosed monovalent antibody fragments to make the claimed pharmaceutical compositions, when Ward, Owens, and the ‘245 patent, either alone or in combination, never even discuss, suggest, or mention instructions for making the claimed antibodies is to assume a level of inspiration constituting inventive activity. The case law makes clear that to avoid a hindsight-based obviousness analysis that the Patent Office bears the

burden of elucidating factual teachings, suggestions, or incentives from the prior art that show the suitability of the combination of references. *See Graham v. John Deere Co.*, 383 U.S. 1, 18, 148 U.S.P.Q. 459, 467 (1966) (“strict observance” of factual predicates to obviousness conclusion required).

Office’s Conclusion Regarding Fab or scFv fragments (claim 66) is Unsupported by Substantial Evidence

Appellants also point out that the Office, provides no authority, as required by the Federal Circuit, for the conclusion that Fab or scFv fragments would “not be expected to cause thrombocytopenia” in the context of an antibody that binds to GP1b. See, for example, *In re Sang Su Lee*, 277 F.3d 1338, 1343, 61 U.S.P.Q.2d 1430, 1433 (Fed. Cir. 2002), *quoting McGinley v. Franklin Sports, Inc.*, 262 F.3d 1339, 1351-1352, 60 U.S.P.Q.2d 1001, 1008 (Fed. Cir. 2001). (“[T]he factual question of motivation [to combine references] is material to patentability, and ...[cannot] be resolved on subjective belief and unknown authority.”).

Office’s Inherency Theory is Legally Incorrect

The Office’s reliance on an inherent characteristic of the resultant antibodies (i.e, “without incurring thrombocytopenia”) is also contrary to Federal Circuit case law. In *In re Rijckaert*, 9 F.3d 1531, 28 U.S.P.Q.2D 1955 (Fed. Cir. 1993), the court faced with an equivalent question unambiguously held that:

“The mere fact that a certain thing may result from a given set of circumstances is not sufficient [to establish inherency.]” *In re Oelrich* , 666 F.2d 578, 581-82, 212 USPQ 323, 326 (CCPA 1981) (citations omitted) (emphasis added). “That which may be inherent is not necessarily known. Obviousness cannot be predicated on what is unknown.” *In re Spormann*, 363 F.2d 444, 448, 150 USPQ 449, 452 (CCPA 1966). Such a retrospective view of inherency is not a substitute for some teaching or suggestion supporting an obviousness rejection. *In re Newell*, 891 F.2d 899, 901, 13 USPQ2d 1248, 1250 (Fed. Cir. 1989)

The holding goes directly to the issue of the appropriateness of the Office’s obviousness rejection in view of its finding that the “resultant antibody fragment ... would not be expected to cause thrombocytopenia.” The Office provides no support for its inherency theory. Appellants’ claim limitation “without incurring thrombocytopenia” is neither “inherent” in Ward and Owens, nor the ‘245 patent. In short, the Office’s retrospective view of inherency is not a substitute for some teaching or suggestion which supports the selection and use of the claimed composition. For this reason too, Appellants respectfully request reconsideration of the obviousness rejection

In conclusion, the Office’s finding of obviousness is neither supported by a scientifically reasoned basis, nor substantial evidence. The Office has not shown a proper *prima facie* case of obviousness, and the rejection of the claims under § 103 for obviousness over Ward in view of Owens and the ‘245 patent should therefore be withdrawn.

Conclusion

Appellants respectfully request that the rejection of claims 65 and 66 be reversed.

Enclosed is a check for \$250.00 in payment of the fee required by 37 C.F.R.

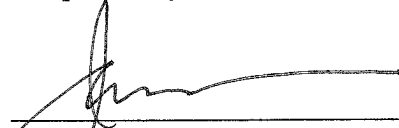
§ 41.20(b)(2).

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Respectfully submitted,

Date:

29 June 2006



James D. DeCamp
Reg. No. 43,580

Clark & Elbing LLP
101 Federal Street
Boston, MA 02110
Telephone: 617-428-0200
Facsimile: 617-428-7045

Claims Appendix A

1-64 (cancelled)

65. (previously presented) A pharmaceutical composition comprising a monovalent antibody fragment which binds *in vivo* to human platelet glycoprotein GPIb without incurring thrombocytopenia and a pharmaceutically acceptable carrier.

66. (previously presented) The pharmaceutical composition according to claim 65, wherein said fragment is a Fab fragment or a single variable domain.

67. (cancelled)

68. (cancelled)

69. (cancelled)

70. (previously presented) The pharmaceutical composition according to claim 65, wherein the variable region of said fragment comprises SEQ ID NO: 4.

71. (previously presented) The pharmaceutical composition according to claim 65, wherein said monovalent antibody fragment is obtained from a monoclonal antibody

produced by the cell line deposited with the Belgian Coordinated Collections of Microorganisms, under accession number LMBP 5108CB.

72. (previously presented) A monovalent antibody fragment which binds *in vivo* to human platelet glycoprotein GPIb, and prevents the binding of von Willebrand factor to human platelet glycoprotein GPIb.

73. (previously presented) The fragment of claim 72, which is an F_{ab} fragment or a single variable domain.

74. (previously presented) The fragment of claim 72, which inhibits platelet adhesion under high shear conditions.

75. (previously presented) The fragment of claim 72, wherein said monovalent antibody fragment is obtained from a monoclonal antibody produced by the cell line deposited with the Belgian Coordinated Collections of Microorganisms, under accession number LMBP 5108CB.

76. (cancelled)

77. (cancelled)

78. (cancelled)

79. (cancelled)

80. (previously presented) A monoclonal antibody produced by the cell line deposited with the Belgian Coordinated Collections of Microorganisms, under accession number LMBP 5108CB.

81. (previously presented) A cell line, capable of producing an antibody directed against GP1b deposited with the Belgian Coordinated Collections of Microorganisms, under accession number LMBP 5108CB.

82. (previously presented) A humanized antibody fragment derivable from the monoclonal antibody of claim 80, wherein said humanized antibody fragment binds GP1b.

83. (previously presented) The antibody fragment of claim 72, wherein the variable regions of said fragment comprises SEQ ID NO: 4.

Claims Appendix B

1-64 (cancelled)

65. (previously presented) A pharmaceutical composition comprising a monovalent antibody fragment which binds *in vivo* to human platelet glycoprotein GPIb without incurring thrombocytopenia and a pharmaceutically acceptable carrier.

66. (previously presented) The pharmaceutical composition according to claim 65, wherein said fragment is a Fab fragment or a single variable domain.

67. (cancelled)

68. (cancelled)

69. (cancelled)

70. (previously presented) The pharmaceutical composition according to claim 65, wherein the variable region of said fragment comprises SEQ ID NO: 4.

71. (previously presented) The pharmaceutical composition according to claim 65, wherein said monovalent antibody fragment is obtained from a monoclonal antibody

produced by the cell line deposited with the Belgian Coordinated Collections of Microorganisms, under accession number LMBP 5108CB.

72. (cancelled)

73. (cancelled)

74. (cancelled)

75. (cancelled)

76. (cancelled)

77. (cancelled)

78. (cancelled)

79. (cancelled)

80. (previously presented) A monoclonal antibody produced by the cell line deposited with the Belgian Coordinated Collections of Microorganisms, under accession

number LMBP 5108CB.

81. (previously presented) A cell line, capable of producing an antibody directed against GP1b deposited with the Belgian Coordinated Collections of Microorganisms, under accession number LMBP 5108CB.

82. (previously presented) A humanized antibody fragment derivable from the monoclonal antibody of claim 80, wherein said humanized antibody fragment binds GP1b.

83. (cancelled)

Evidence Appendix

Cadroy *et al.*, *Blood* 83:3218-3224 (1994) – Final Office Action mailed May 27, 2005, page 7

Bergmeier *et al.*, *Blood* 95:886-893 (2000) – Final Office Action mailed May 27, 2005, page 7

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Relative Antithrombotic Effects of Monoclonal Antibodies Targeting Different Platelet Glycoprotein-Adhesive Molecule Interactions in Nonhuman Primates

By Yves Cadroy, Stephen R. Hanson, Andrew B. Kelly, Ulla M. Marzec, Bruce L. Evatt, Thomas J. Kunicki, Robert R. Montgomery, and Laurence A. Harker

The relative antithrombotic effectiveness of targeting glycoprotein (GP) Ib-dependent versus GPIIb-IIIa-dependent platelet interactions has been determined in baboons by measuring thrombus formation after infusing comparable antithrombotic doses of anti-von Willebrand factor (vWF) monoclonal antibody (MoAb) BB3-BD5, anti-GPIb MoAb AP1, and anti-GPIIb-IIIa MoAb LJ-CP8 under conditions of arterial and venous flow (shear rates of 750 to 1,000 seconds⁻¹ and 100 seconds⁻¹, respectively). Thrombus formation was quantified as ¹¹¹In-platelet deposition and ¹²⁵I-fibrin accumulation on segments of collagen-coated tubing interposed in chronic exteriorized arteriovenous (AV) shunts for 40 minutes. In vitro, anti-vWF MoAb BB3-BD5 (IgG) and anti-GPIb MoAb AP1 (IgG or F(ab)₂ fragments) inhibited ristocetin-induced platelet aggregation (IC₅₀ 50 nmol/L and 1 μmol/L, respectively), but neither of these MoAbs blocked platelet aggregation induced by adenosine diphosphate (ADP) (P > .5). Conversely, anti-GPIIb-IIIa MoAb LJ-CP8 inhibited platelet aggregation induced by ADP (IC₅₀ 1 μmol/L), but failed to block ristocetin-induced platelet aggregation (P > .5). In vivo, the intravenous infusion of anti-vWF MoAb BB3-BD5 or anti-GPIIb-IIIa MoAb LJ-CP8 into baboons at

doses that abolished corresponding agonist-induced aggregation ex vivo (bolus injections of 0.5 mg/kg and 10 mg/kg, respectively) prolonged template bleeding times from baseline values of 4.0 ± 0.3 minutes to >27 ± 4 minutes, and to >25 ± 4 minutes, respectively (P < .001 in both cases), without affecting the peripheral platelet count (P > .5). However, injection of anti-GPIb MoAb AP1 (10 mg/kg as IgG or 1 mg/kg as F(ab)₂ fragments) produced immediate irreversible thrombocytopenia (<40,000 platelets/μL). Anti-GPIIb-IIIa MoAb LJ-CP8 abolished platelet deposition and fibrin accumulation on collagen segments under both arterial and venous flow conditions (P < .01 in all cases), whereas MoAb BB3-BD5 produced minimal inhibition of platelet deposition and no decrease in fibrin accumulation at arterial shear rates and undetectable antithrombotic outcomes at low shear. Thus, inhibiting GPIIb-IIIa-dependent platelet recruitment abrogates both thrombus formation and platelet hemostatic function at both venous and arterial shear rates. By contrast, interfering with GPIb-vWF-dependent platelet interactions abolishes platelet hemostatic function without producing corresponding antithrombotic effects.

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Platelet adhesion and cohesion are essential processes in hemostatic plug formation and vascular thrombus formation. These reactions are mediated by platelet membrane glycoprotein (GP)-receptor binding with adhesive proteins, including fibrinogen, von Willebrand factor (vWF), fibronectin, vitronectin, laminin, and thrombospondin. Under high-shear flow conditions the initial attachment of platelets to subendothelial matrix proteins depends largely on platelet GPIb binding with vWF.^{1,2} For example, in flow chambers anti-GPIb monoclonal antibody (MoAb) or anti-vWF MoAb directed against the corresponding binding domains of vWF or GPIb inhibit both platelet adhesion and accumulation on subendothelium and collagen-coated surfaces.^{1,2} In vivo,

MoAbs against GPIb markedly prolong the bleeding time in swine,³ and anti-vWF MoAbs exhibit both antihemostatic and antithrombotic effects in the same species.^{7,8}

Interactions with adhesive proteins and expressed GPIIb-IIIa receptors are critical for platelet recruitment in the formation of thrombus,⁹ as illustrated by the capacity of MoAbs directed against GPIIb-IIIa to impair platelet spreading and thrombus formation on subendothelium, particularly under arterial flow conditions.^{3,5,10} In experimental animals these MoAbs against GPIIb-IIIa are substantially more antithrombotic than aspirin and standard heparin.¹¹⁻¹⁴ Such MoAbs also interfere with aspirin- and heparin-resistant thrombotic events in patients undergoing thrombolytic therapy for acute myocardial infarction and unstable angina.^{15,16}

To determine the relative antithrombotic efficacy for anti-vWF-GPIb MoAb and anti-GPIIb-IIIa MoAb at equivalent antihemostatic doses in the same model system in vivo, thrombus formation has been compared in baboons after intravenous administration of (1) anti-vWF MoAb BB3-BD5, (2) anti-GPIb MoAb AP1, and (3) anti-GPIIb-IIIa MoAb LJ-CP8. The baboon was selected as the experimental animal model because the hemostatic apparatus in this species is similar to that in humans.¹⁷ Platelet hemostatic function was assessed in vivo by standard template bleeding time measurements, and ex vivo by platelet aggregation induced by ristocetin and adenosine diphosphate (ADP).¹¹ Thrombus formation was measured in real time as the deposition of ¹¹¹In-platelets by gamma camera imaging and accumulation of ¹²⁵I-fibrin on segments of collagen-coated tubing interposed in exteriorized femoral arteriovenous (AV) shunts in baboons.¹⁸ To assess the relative dependency of these processes on flow conditions, thrombus formation onto collagen segments was compared for venous flow (blood flow of 20

From the Division of Hematology and Oncology and Yerkes Regional Primate Research Center, Emory University School of Medicine; Centers for Disease Control, Atlanta, GA; Laboratoire Central D'Hématologie-Hémostase, Hôpital Purpan, Toulouse, France; the Department of Molecular and Experimental Medicine, Scripps Research Institute, La Jolla, CA; and The Blood Center of Southeastern Wisconsin, Milwaukee.

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Address reprint requests to Laurence A. Harker, MD, Division of Hematology and Oncology, Emory University School of Medicine, PO Drawer AR, Atlanta, GA 30322.

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mL/min with wall shear rate of 100 seconds⁻¹) and flow encountered in medium-sized arteries (blood flow of 150 to 200 mL/min producing wall shear rates of 750 to 1,000 seconds⁻¹).

MATERIALS AND METHODS

Animal studies. Eleven normal male baboons (*Papio anubis*), weighing 9 to 11 kg, were used. All procedures were approved by the Institutional Animal Care and Use Committee in accordance with Federal Guidelines (Guide for the Care and Use of Laboratory Animals). The baboons were dewormed and observed to be disease free for at least 6 weeks. As previously described, all animals had a chronic exteriorized arteriovenous shunt of silicone rubber tubing surgically placed between the femoral artery and vein. These chronic shunts have previously been shown not to detectably shorten platelet survival or produce measurable platelet activation.^{19,20} The peripheral blood perfusing the AV shunts was subject to normal dilution, filtration, and inactivation mechanisms in the host animals.

Thrombus was formed on 2-cm tubular segments (3.2-mm internal diameter) covalently coated with type I collagen as previously described.¹⁹ The collagen coating was uniform and was not removed by extensive washing.¹⁹ Two collagen-coated cannular segments were interposed in separate parallel arms incorporated as extension segments into the chronic exteriorized AV shunt and exposed to circulating native blood under arterial versus venous flow conditions for 40 minutes.²¹ Blood flow was maintained at 150 to 200 mL/min in the arterial arm of the AV shunt using a clamp placed distal to the collagen-coated segment, and the flow rate in the parallel arm of the shunt system was held at 20 mL/min using a peristaltic roller pump (Model 7016; Cole-Parmer, Chicago, IL) also placed distal to the thrombogenic collagen-coated segment. Flow rates in the arms of the AV shunt were continuously measured using ultrasonic flowmeters (Transonic Systems, Inc, Ithaca, NY). Initial wall shear rates were calculated, assuming Poiseuille flow, to be 750 to 1,000 seconds⁻¹ under high-flow conditions (150 to 200 mL/min) and 100 seconds⁻¹ under low-flow conditions (20 mL/min).

Thrombus formation on the collagen segments was measured as (1) the deposition of autologous ¹¹¹In-platelets in real time using scintillation camera imaging, and (2) the accumulation of ¹²⁵I-fibrin.¹⁹ Platelets were labeled with ¹¹¹In-oxine using a previously described technique.¹⁹ The average labeling efficiency was greater than 90%. Platelet accumulation was measured with a Picker DC 4/11 Dyna scintillation camera (Picker Corp, Northford, CT) interfaced with a Medical Data System A³ image processing system (Medtronic, Ann Arbor, MI). Dynamic images were acquired at 5-minute intervals over a 40-minute study period. The total number of deposited platelets (labeled plus unlabeled cells) was calculated as performed previously.^{19,20}

Fibrin deposition was determined using injected ¹²⁵I-fibrinogen. Baboon fibrinogen was purified using β -alanine precipitation and homologous fibrinogen preparations were labeled with ¹²⁵I using the ICI method as described.¹⁹ Labeling efficiencies averaged 70%; thrombin clottability of the labeled fibrinogen was greater than 90%. ¹²⁵I-fibrinogen, 5 μ Ci, was injected intravenously 10 minutes before interposing the parallel thrombogenic devices into the chronic AV shunt. At the end of the 40-minute study period, the collagen-coated tubing was removed and thoroughly washed with isotonic saline. Total fibrin deposition (labeled plus unlabeled protein) was calculated after allowing the ¹¹¹In activity to decay (half-life 2.8 days) for 30 days, as previously described.¹⁹

Bleeding times were performed on the shaved volar surface of the forearm using the standard template method as previously reported.²² Results of greater than 30 minutes were assigned a 30-minute value.

Antibody preparation and characterization. Anti-vWF MoAb

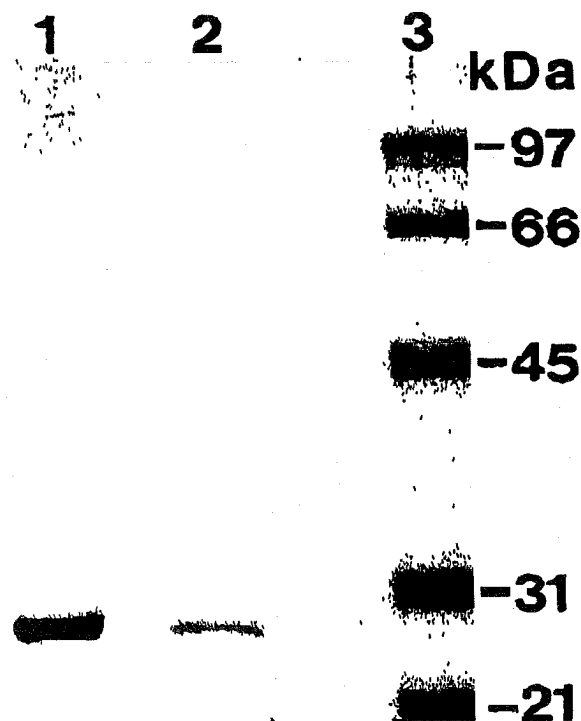


Fig 1. Immunoreactivity of MoAb BB3 BD5 for the GPIb binding domain of vWF. Electrophoresis (10% SDS-PAGE) of a recombinant 25-kD GPIb binding domain of vWF (VCL) is shown stained for protein in lane 2. MoAb BB3 BD5 binds with the 25-kD GPIb binding domain of vWF in lane 1. Protein markers for protein size are shown in lane 3.

BB3 BD5 belongs to the IgG₁ subclass.⁷ Purified IgG was prepared from ascitic fluid using protein A Sepharose. The material was homogeneous as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), exhibiting a single band at 150,000 Mr. In vitro MoAb BB3 BD5 abolished ristocetin-induced baboon platelet aggregation (ristocetin 1.24 mg/mL final concentration) at a concentration of 5 μ g/mL (see below). To establish its specificity for the platelet GPIb binding domain of vWF, immunoblotting was performed (Fig 1). In these experiments 10% SDS-PAGE was performed for 10 μ g of recombinant peptide comprising residues Leu504-Lys728 of native GPIb plus an aminoterminal methionine but including the single interchain disulfide bond linking residues Cys509 and Cys695 (designated VCL, a gift from Bio-Technology General [Israel] Ltd, Rehovot, Israel).²³ After electrophoresis, VCL was transferred from the gel onto nitrocellulose membrane by wet electrophoretic transfer. MoAb BB3 BD5 (5 and 50 μ g) was incubated with the membrane, washed and detected with alkaline phosphatase-labeled antimurine IgG antibody, and compared with direct staining of VCL subjected to concurrent electrophoresis in association with molecular marker proteins (Fig 1).

Anti-vWF MoAb API, an MoAb of the IgG₁ subclass, is directed against platelet receptor GPIb.²⁴ IgG was purified from ascitic fluid using protein A Sepharose. In vitro, MoAb API eliminated ristocetin-induced baboon platelet aggregation at 100 μ g/mL final concentration. Bivalent F(ab)₂ fragments were prepared from AP-1 IgG by papain digestion.²⁴

Anti-GPIIb-IIIa MoAb LJ-CP8 is an MoAb of the IgG₁ subclass directed against platelet GPIIb-IIIa.¹¹ MoAb LJ-CP8 was a gift from Dr Zavarico M. Ruggeri (Scripps Research Institute, La Jolla, CA), provided by Rhône-Poulenc-Rorer (Collegeville, PA). In vitro, MoAb LJ-CP8 inhibited baboon fibrinogen binding to thrombin-stimulated baboon platelets, and abolished ADP-induced baboon platelet aggregation at 100 µg/mL final concentration.

MoAb BB3 BD5 and MoAb LJ-CP8 were administered intravenously at doses attaining circulating levels that saturate the respective epitopes and abolished corresponding agonist platelet aggregation in vitro, ie, 5 µg/mL and 100 µg/mL, respectively.

Laboratory procedures. For counting platelets, leukocytes, and erythrocytes, whole blood was collected in vacutainer tubes (Becton Dickinson, Rutherford, NJ) containing diadodium EDTA and enumeration was performed using an Automated Cell Counter (System 9000; Beker Instrument Corp, Allentown, PA).

Platelet aggregation studies were performed on platelet-rich plasma (PRP) prepared from citrated blood (9 vol blood into 1 vol 3.2% sodium citrate) using an aggregometer (Chronolog, Havertown, PA) as reported elsewhere.¹¹ The platelet concentration was adjusted to 250,000 platelets/µL. The agonists used were ADP (Sigma Chemical Co, St Louis, MO) and ristocetin (Sigma). The control results were expressed as the agonist concentration required to induce a half-maximal increase in light transmission (AC₅₀) through the stirred suspension of PRP. When the MoAbs prevented platelet aggregation at 77 µmol/L ADP and 1.32 mg/mL ristocetin, aggregation was considered to be "abolished" for these agonists.

von Willebrand ristocetin cofactor activity (vWF:RCo) in plasma was determined by measuring the ability of studied plasmas to agglutinate formalin-fixed platelets in the presence of 1.24 mg/mL ristocetin (Ristocetin Cofactor Assay Kit; Helena Laboratories, Beaumont, TX) and expressed as percentage of value obtained from a pool of control normal baboons (n = 8). Factor VIII coagulant activity (FVIII:C) was measured using a chromogenic assay (Stachrom VIII:C assay; Stago, Asnières, France), and expressed as percentage of the value obtained from a pool of control normal baboons (n = 8).

Statistical analysis. All data are shown as the mean (±1 SE). Statistical analyses were performed using the CLINFO system of the US Department of Health and Human Services. The Student's *t*-test (two-tailed) for unpaired samples group was used to assess significance.

RESULTS

Effects of anti-GPIb MoAb AP1 on peripheral platelets. The bolus intravenous injection of MoAb AP1 IgG (10 mg/kg) destroyed peripheral platelets within 5 minutes, ie, the platelet count decreased irreversibly from 382,000 platelets/µL to less than 40,000 platelets/µL. To determine whether platelet destruction was dependent on the Fc portion of the antibody, MoAb AP1 F(ab)₂ (1 mg/kg) was prepared and subsequently administered. The platelet count decreased from 360,000 platelets/µL to <40,000 platelets/µL immediately after the injection of MoAb AP1 F(ab)₂. Consequently, no additional in vivo studies were performed with MoAb AP1.

Effects of anti-vWF MoAb BB3 BD5 on hemostasis and thrombosis. To establish directly the intravenous dose of MoAb BB3 BD5 capable of abolishing platelet hemostatic function in baboons, template bleeding time and ristocetin aggregation ex vivo were determined in a dose-ranging study. Fifteen minutes after administering bolus injections of MoAb BB3 BD5 (0.1, 0.3, and 0.5 mg/kg), template

Table 1. Effect on Hemostasis of Anti-vWF MoAb BB3 BD5 and Anti-GPIIb-IIIa MoAb LJ-CP8

	Control	MoAb BB3 BD5	MoAb LJ-CP8
Platelet count (×10 ³ /µL)	385 ± 44	360 ± 32	430 ± 35
Hematocrit (%)	32 ± 1	30 ± 1	39 ± 1
Fibrinogen level (g/L)	3.2 ± 0.2	3.6 ± 0.3	3.3 ± 0.5
Bleeding time (min)	4.0 ± 0.3	>27 = 4	>26 = 4
AC ₅₀ ADP (µmol/L)	3.0 ± 0.3	2.7 ± 0.6	Abolished
AC ₅₀ ristocetin (mg/mL)	1.0 ± 0.1	Abolished	1.1 ± 0.1
vWF:RCo (%)	115 ± 15	<6	107 ± 7
FVIII:C (%)	95 ± 11	98 ± 18	102 ± 15

Blood sampling for performing assays in vitro, and the bleeding time determinations were performed 15 minutes after administering the MoAbs. Each mean represents the average of five animals.

bleeding times were prolonged in a dose-dependent manner from the baseline value of 4.5 ± 1.2 minutes to 7.8 ± 2.2 minutes, 10.5 ± 2.6 minutes, and >30 minutes, respectively (n = 3). Ex vivo ristocetin-induced platelet aggregation was similarly inhibited in a dose-response fashion by bolus injections of the MoAb BB3 BD5, ie, maximum aggregation induced by 1.24 mg/mL ristocetin was reduced from the baseline of 78% to 56%, 12%, and 0%, respectively. On the basis of these findings, intravenous bolus injections of 0.5 mg/kg MoAb BB3 BD5 were used in studies comparing antithrombotic outcomes.

The effects on hemostasis of infusing MoAb BB3 BD5 (0.5 mg/kg) intravenously into five animals are shown in Table 1. Within 15 minutes of injecting MoAb BB3 BD5 ristocetin-induced platelet aggregation ex vivo was abolished, and vWF:RCo activity remained defective for at least 6 hours, but returned to baseline by 24 hours. The template bleeding time was also markedly prolonged by MoAb BB3 BD5, ie, from 4.0 ± 0.3 minutes to >27 ± 4 minutes, and returned to baseline within 24 hours. Neither ADP-induced platelet aggregation nor FVIII:C were diminished by injections of MoAb BB3 BD5.

The results of measuring platelet deposition and fibrin accumulation onto segments of collagen-coated tubing at two different shear rates are presented in Figs 2 and 3. Ten minutes after injecting MoAb BB3 BD5, parallel thrombogenic collagen segments were incorporated into the exteriorized AV shunt. Forty minutes later, MoAb BB3 BD5 reduced platelet deposition from 2.42 ± 0.38 × 10⁶ platelets in control studies to 1.37 ± 0.30 × 10⁶ platelets (P = .06) at arterial shear rates (750 to 1,000 seconds⁻¹; Fig 2), but at low shear rates (100 seconds⁻¹) MoAb BB3 BD5 failed to decrease thrombus formation (Fig 2; 1.3 ± 0.14 × 10⁶ platelets v 1.8 ± 0.26 × 10⁶ platelets; P = .19). Furthermore, fibrin deposition was not decreased by the anti-vWF antibody at either arterial or venous blood flow (Figs 2 and 3; P > .35 in both cases).

Effect of anti-GPIIb-IIIa MoAb LJ-CP8 on hemostasis and thrombosis. To document the intravenous dose of MoAb LJ-CP8 able to abrogate platelet hemostatic function in baboons, dose-ranging studies with injected MoAb were performed, measuring template bleeding times and ADP-induced aggregation ex vivo. Fifteen minutes after adminis-

tering bolus injections of MoAb LJ-CP8 (2, 5, and 10 mg/kg), template bleeding times were prolonged from the baseline value of 4.0 minutes to 10.5 minutes, 22 minutes, and >30 minutes, respectively ($n = 2$). Ex vivo ADP-induced platelet aggregation was similarly impaired in a dose-response manner by these increasing bolus doses of the MoAb, ie, percent of maximum baseline aggregation induced by 10 $\mu\text{mol/L}$ ADP was reduced from 100% to 63%, 20%, and 0%, respectively. Accordingly, intravenous bolus injections of 10 mg/kg MoAb LJ-CP8 were used in studies assessing antithrombotic outcomes.

Ex vivo ADP-induced platelet aggregation was abolished 15 minutes after administering MoAb LJ-CP8 (10 mg/kg) into five baboons, and this effect lasted for greater than 48 hours. Anti-GPIIb-IIIa MoAb LJ-CP8 also prolonged the bleeding time from 4.0 ± 0.3 minutes to $>26 \pm 4$ minutes, and the bleeding times returned to normal by 72 hours (Table 1). Neither ristocetin-induced platelet aggregation (vWF:RCO) nor FVIII:C levels were reduced by MoAb LJ-CP8 administration (Table 1).

At both arterial and venous blood flows MoAb LJ-CP8 abolished platelet deposition onto collagen-coated segments (Figs 2 and 3; $P < .01$ in both cases). Fibrin accumulation was also markedly reduced, although the reduction in fibrin deposition by MoAb LJ-CP8 was detectably greater at the high shear rate, ie, 0.04 ± 0.02 mg versus control of 0.49 ± 0.12 mg ($P = .03$), compared with 0.30 ± 0.07 mg versus control of 0.60 ± 0.09 mg for the lower shear rate ($P = .03$). The effects of anti-GPIIb-IIIa MoAb LJ-CP8 versus anti-vWF MoAb BB3 BD5 on thrombus formation were significantly different at arterial shear rates ($P < .05$), and strikingly different at low shear rates ($P < .001$).

DISCUSSION

The present study shows that in nonhuman primates platelet GPIIb-IIIa receptors mediate platelet recruitment in the

process of thrombus formation under both arterial and venous flow conditions, whereas vWF-GPIb interactions make no significant contribution to thrombus forming at low shear and contribute little at shear rates characteristically found in medium-sized arteries. By contrast, platelet hemostatic function is dependent on both vWF-GPIb interactions and GPIIb-IIIa-mediated recruitment.

Determining the relative antithrombotic activity of MoAbs targeting different platelet glycoprotein-adhesive molecule interactions depends, in part, on the thrombosis model used, and may therefore vary according to the clinical focus of the thrombotic process being modeled. The model system used in this study measures the formation of thrombus as the deposition of ^{125}I -platelets and ^{125}I -fibrin on segments of collagen-coated tubing interposed in exteriorized femoral AV shunts under conditions of controlled blood flow.^{18,21} This model system is independent of heparin effects, as shown by the fact that anticoagulating doses of unfractionated heparin do not lessen thrombus formation on these collagen-coated segments.¹⁸ Because of the established importance of shear rate in assessing platelet adhesion and cohesion,^{2,3,8,10,22} we strictly controlled flow conditions in the present study, ie, intermediate shear rate (750 to 1,000 seconds⁻¹) corresponding to flow conditions seen in medium- and small-size arteries, and low-shear rate (100 seconds⁻¹) characteristically found in veins.

Anti-vWF MoAb BB3 BD5 inhibits vWF-dependent platelet adhesion and cohesion by blocking the GPIb binding domain of vWF (Fig 1). When MoAb BB3 BD5 (0.5 mg/kg) is injected into baboons, hemostatic plug formation and ristocetin-induced platelet aggregation ex vivo are abrogated without inhibiting platelet aggregation induced by ADP or FVIII:C activity (Table 1). Based on baboon blood volumes of 70 mL/kg, this dose produces peak blood levels that exceed concentrations needed to eliminate ristocetin-induced platelet aggre-

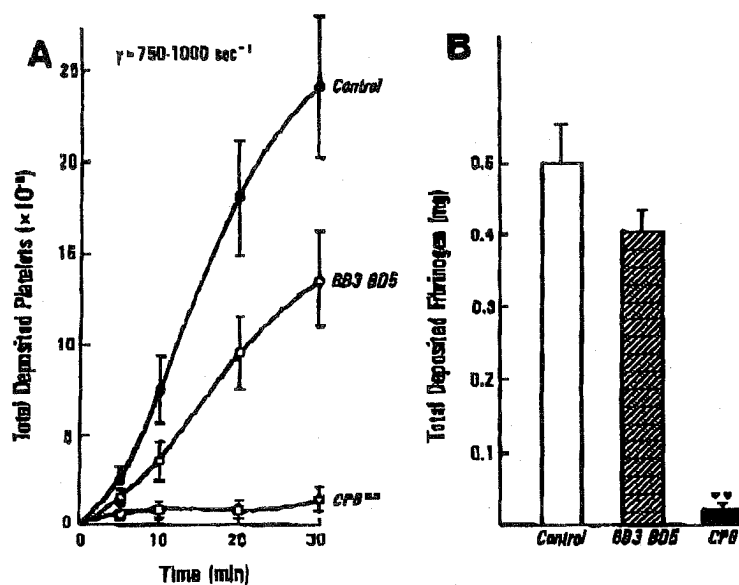


Fig 2. Effect of anti-vWF MoAb BB3 BD5 and anti-GPIIb-IIIa MoAb LJ-CP8 on thrombus formation initiated by collagen-coated segments under high-shear rate blood flow conditions (wall shear rate 750 to 1,000 seconds⁻¹). (A) MoAb LJ-CP8 reduced platelet accumulation over 30 minutes of blood exposure more effectively ($P = .007$ v control values) than MoAb BB3 BD5 ($P = .06$ v control values). (B) MoAb LJ-CP8 abolished fibrin accumulation after 30 minutes of blood exposure ($P = .028$ v control values), whereas MoAb BB3 BD5 was ineffective ($P > .5$ v control values).

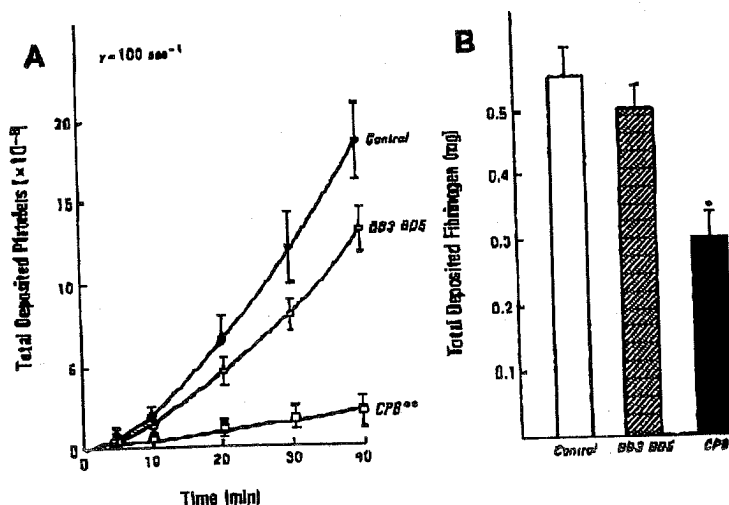


Fig 3. Effect of anti-vWF MoAb BB3 BD5 and anti-GPIIb-IIIa MoAb LJ-CP8 on thrombus formation initiated by collagen-coated segments under low-shear rate blood flow conditions (wall shear rate 100 s^{-1}). (A) Platelet accumulation over 40 minutes of blood exposure was interrupted by MoAb LJ-CP8 ($P = .006$ v control values), but only slightly reduced by MoAb BB3 BD5 ($P = .19$ v control values). (B) Fibrin accumulation after 40 minutes of blood exposure was reduced by MoAb LJ-CP8 ($P = .025$ v control values), but unaffected by MoAb BB3 BD5 ($P > .35$ v control values).

gation in vitro, ie. $5 \mu\text{g/mL}$. Despite this profound antithrombotic effect of MoAb BB3 BD5, detectable antithrombotic effects are restricted to arterial shear conditions.

MoAb LJ-CP8 inhibits platelet recruitment by blocking interactions between adhesive proteins and functional GPIIb-IIIa receptors expressed by activated platelets.^{11,26,27} Intravenous injections of MoAb LJ-CP8 (10 mg/kg) into baboons interrupt platelet-dependent thrombus formation, and abolish hemostatic plug formation and ADP-induced platelet aggregation ex vivo, without altering ristocetin-induced platelet aggregation or FVIII:C activity (Figs 2 and 3; Table 1). Thus, at equivalent antithrombotic doses, MoAb LJ-CP8 is substantially more potent than MoAb BB3 BD5 in preventing platelet deposition on collagen-coated segments at both arterial and venous shear rates ($P = .020$ at high-shear rate, and $P = .0007$ at low-shear rate). Antithrombotic comparability of the administered doses for the two MoAbs was verified by dose-response bleeding time and ADP- and ristocetin-induced platelet aggregation studies ex vivo, together with concordant platelet aggregation data in vitro. However, these data do not address directly the postulate that vWF-GPIb-dependent platelet interactions are important in the formation of thrombus at sites of severe arterial stenosis exhibiting very high shear rates. However, because the blood levels achieved by the administered doses of MoAb LJ-CP8 and MoAb BB3 BD5 saturated GPIIb-IIIa epitopes and vWF binding sites for GPIb, respectively, higher doses of MoAb BB3 BD5 are unable to produce greater antithrombotic effects under the experimental conditions used in this report.

Anti-GPIIb-IIIa MoAb LJ-CP8 is known to prevent aspirin- and heparin-resistant thrombus formation on thrombogenic devices interposed either as segments of Dacron vascular graft in exteriorized AV shunts,¹¹ or as surgical endarterectomies or implanted Gore-Tex (W.L. Gore, Flagstaff, AZ) grafts in carotid arteries.²⁷ The present study extends the previous findings by comparing the relative effects of these MoAbs on thrombus formation induced by collagen, a thrombogenic substrate relevant to vascular injury, with

shear rate as an independent variable. Clearly, the results of the present study confirm the antithrombotic efficacy of targeting platelet GPIIb-IIIa for a broad range of shear rates.

As opposed to the striking antithrombotic effects of MoAb LJ-CP8, anti-vWF MoAb BB3 BD5 has no antithrombotic efficacy at venous shear rates and produces only minimal antithrombotic effects at shear rates found in clinically relevant-sized arteries (Figs 2 and 3). Anti-vWF MoAb BB3 BD5 has been reported to have greater antithrombotic effects than aspirin in a porcine model of coronary artery stenosis.^{7,8} Presumably, the disparate outcome in their study using anti-vWF MoAb BB3 BD5 with stenotic arteries compared with the results using collagen segments in the present report is explained by the very high shear rates present in damaged stenotic coronary arteries. The finding in the present report that anti-vWF MoAb BB3 BD5 exhibits detectable antithrombotic effects restricted to higher shear conditions (Figs 2 and 3) is consistent with this interpretation.^{3,23} In addition, studies using collagen flow chamber in vitro indicate that combining MoAbs directed against vWF domains interacting with GPIb, GPIIb-IIIa, and collagen greatly enhances antithrombotic outcomes.⁴

Of additional interest in the present study is the observation that anti-GPIb MoAb AP1 injected intravenously as whole IgG into baboons destroys circulating platelets, leading to acute severe thrombocytopenia, and that the infusion of F(ab)₂ fragments produces equivalent thrombocytopenia. There are a number of possible explanations for acute Fc-independent antibody-induced thrombocytopenia induced by MoAb AP1. First, antibody-induced platelet activation may produce platelet aggregates that clear quickly from the circulation. In this regard, MoAb AP1 rapidly induces baboon platelet aggregation in vitro that approximates 15% to 20% of maximum achievable aggregation induced by physiologic agonists (unpublished observations). Second, GPIb may be important for platelet viability. For example, patients with autoantibodies against platelet GPIb usually develop immune thrombocytopenic purpura.²⁸ This argument may be

negated by the observation that platelet counts do not decrease after injecting anti-GPIIb MoAb PP4-3B into pigs.⁶ Third, baboons may have naturally occurring antibodies that cross-react with murine MoAb AP1, initiating immune complex-dependent platelet clearance.^{2a} Finally, some as-yet-to-be described Fe-independent mechanism may produce platelet immune destruction.

Previously it has also been shown that certain anti-GPIIb-IIIa MoAbs also decrease peripheral platelet counts.^{11,2a} For example, the injection of anti-GPIIb-IIIa MoAb AP2 into baboons reduces the platelet count by about half within 2 hours, but without initiating platelet granular release in vivo.¹¹ Moreover, the anti-GPIIb-IIIa MoAb LJ-CP8 used in the present study causes severe but delayed platelet destruction, giving rise to transient thrombocytopenia. Platelet counts begin to decrease 4 to 5 days after injecting the antibody, reach a nadir of <30,000 platelets/ μ L on days 5 to 6, and return to normal thereafter.^{2a} We interpret the delayed thrombocytopenia after the infusion of MoAb LJ-CP8 to reflect the production of endogenous antibody against the injected murine IgG. Thus, these earlier studies as well as the present report show the importance of measuring platelet count when evaluating the antithrombotic and antihemostatic effects of antiplatelet antibodies, because antibody injections may be complicated by acute (MoAb AP1) or delayed (MoAb LJ-CP8) severe thrombocytopenia. Because thrombocytopenia would preclude the use of specific antibodies in humans, natural or synthetic peptides inhibiting platelet GPIIb-IIIa-dependent recruitment may ultimately be therapeutically more useful.^{2a}

In summary, the present study shows that blocking platelet GPIIb-IIIa using MoAb LJ-CP8 potently interrupts thrombus formation over a wide range of shear rates, whereas anti-vWF MoAb BB3 BD5 produces minimal antithrombotic effects restricted to higher shear conditions. However, both of these MoAbs abolish platelet hemostatic function.

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Structural and functional characterization of the mouse von Willebrand factor receptor GPIb-IX with novel monoclonal antibodies

Wolfgang Bergmeier, Kirsten Rackebandt, Werner Schröder, Hubert Zirngibl, and Bernhard Nieswandt

Five novel monoclonal antibodies (mAbs; p0p 1-5) were used to characterize the structural and functional properties and the *in vivo* expression of the murine GPIb-IX complex (von Willebrand factor receptor). The molecular weights of the subunits are similar to the human homologs: GPIb α (150 kd), GPIb β (25 kd), and GPIX (25 kd). Activation of platelets with thrombin or PMA predominantly induced shedding of glycocalcin (GC; 130 kd) but only low levels of receptor internalization.

The GC concentration in normal mouse plasma was found to be at least 10 times higher than that described for human plasma (approximately 25 μ g/mL versus 1-2 μ g/mL). Two additional cleavage sites for unidentified platelet-derived proteases were found on GPIb α , as demonstrated by the generation of 3 N-terminal fragments during *in vitro* incubation of washed platelets (GC, 60 kd, 45 kd). Occupancy of GPIb α with p0p mAbs or F(ab)₂-fragments resulted in aggregate formation *in vitro* and rapid irreversible

thrombocytopenia *in vivo*, irrespective of the exact binding epitopes of the individual antibodies. GPIb-IX was not detectable immunohistochemically on endothelial cells in the major organs under normal or inflammatory conditions. The authors conclude that the mouse system might become an interesting model for studies on GPIb-IX function and regulation. (Blood. 2000;95:888-893)

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Introduction

Platelet adhesion to sites of vascular injuries is mediated by the interactions of glycoprotein (GP) membrane receptors on circulating platelets with their distinct adhesive ligands.¹ In particular, under high shear stress, the platelet GPIb-IX-V receptor complex contributes to this process initiating hemostasis through interactions with the adhesive ligand von Willebrand factor (vWF).^{2,4} The human GPIb-IX-V complex consists of 4 distinct gene products: GPIb α , Ib β , IX, and V.^{5,6} Unstimulated platelets express approximately 23 000 copies of GPIb-IX and 12 000 copies of GPV on their surfaces.⁶ Binding sites for vWF and α -thrombin have been identified on the N-terminal 45-kd region of GPIb α .^{7,9} Interactions with vWF molecules only occur when the latter are bound to subendothelium,¹⁰ fibrin,¹¹ or collagen,¹² and they require high shear forces.¹³ Interactions are also detectable during ristocetin- or botrocetin-induced platelet agglutination, probably because of neutralization of the repulsive negative charges by the positively charged ristocetin or botrocetin molecules.¹⁴⁻¹⁶ Subsequent activation of the fibrinogen receptor GPIIb/IIIa leads to the formation of stronger bonds and therefore platelet adhesion and aggregation.^{4,17} In contrast, other platelet agonists such as adenosine diphosphate, collagen, or thrombin induce the binding of vWF to GPIIb/IIIa.^{18,19} The effect of platelet activation on the surface expression of GPIb-IX *in vitro* has been a matter of controversy. Although most investigators demonstrate the translocation of GPIb-IX-complexes from the plasma membrane to the surface-connected canalicular system in response to thrombin (without receptor shedding),²⁰⁻²² significant shedding has been observed by others.²³ Furthermore, White et al²⁴ found no effect of thrombin activation on GPIb-IX surface expression. In con-

trast, it is commonly accepted that GPIb α can be proteolyzed by neutrophil cathepsin G,²⁵ neutrophil elastase,²⁶ or platelet calpain.²⁷

The platelets of patients with Bernard-Soulier syndrome, a congenital bleeding disorder, show diminished agglutination to vWF in the presence of ristocetin and a reduced response to low doses of thrombin. This results from a reduced expression or a malfunction of GPIb-IX²⁸ that coincides with the release of "giant" platelets,^{1,2} indicating a role of the GPIb-IX-V complex in maintaining circulating platelet morphology. Reports describing the expression of GPIb α or even all subunits of the receptor complex on cells of nonhematopoietic origin, particularly endothelial cells (EC), raised speculations about unrecognized functions of GPIb-IX-V.²⁹⁻³¹ However, these data are not without controversy because others could not reproduce the findings.³² No systematic investigations of GPIb-IX expression on a cellular level *in situ* have been performed to date.

As proposed recently,³³ the vWF-receptor complex may become an interesting pharmacologic target for the prevention of thrombotic and inflammatory complications. Because *in vivo* investigations are obviously limited in humans and nonhuman primates, there is a need for small animal models allowing for *in vivo* studies on GPIb-IX-V functions under normal and inflammatory conditions. In the mouse system, adequate animal models exist, but limited information about structure, function, and regulation of the murine receptor complex has been available. In the current study, we investigated the structural and functional proper-

From the Department of Molecular Oncology, General Surgery, University of Witten-Herdecke, and the BAYER Pharma Research Center, Wuppertal, Germany.

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Reprints: Bernhard Nieswandt, IMMI, Klinikum Wuppertal, Universität Witten-

Herdecke, Heusenstrasse 40, D-42283 Wuppertal, Germany; e-mail: nieswandt@klinikum-wuppertal.de.

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ties of mouse GPIIb-IX and examined the *in vivo* expression of the complex with novel monoclonal antibodies.

Materials and methods

Animals

Specific-pathogen-free mice (NMRI, BALB/c) 6 to 10 weeks of age were obtained from Charles River (Sulzfeld, Germany) and kept in our animal facilities.

Reagents

EZ-Link sulfo-NHS-LC-biotin (Pierce, Rockford, IL), immobilized pepsin (Pierce), ristocetin (EUROPA, Cambridge, UK), phorbol 12-myristate 13-acetate (PMA; Sigma, Deisenhofen, Germany), high molecular weight heparin (Sigma), thrombin (Boehringer Mannheim, Mannheim, Germany), Collagen A1 (Biochrom, Berlin, Germany), and streptavidin-horse radish peroxidase (HRP; DAKO, Glostrup, Denmark) were purchased. Lipopolysaccharide (LPS from *Salmonella minnesota* 9700) was obtained from Difco Laboratories (Detroit, Michigan).

Antibodies

Rat anti-mouse P-selectin mAb RB40J4 was kindly provided by D. Vestweber (Münster, Germany). Polyclonal rabbit antibodies to human fibrinogen and vWF were purchased from DAKO and were modified in our laboratories. Rabbit anti-fluorescein isothiocyanate (FITC)-HRP and rabbit anti-rat Ig-FITC were purchased from DAKO. All other antibodies were generated, produced, and modified in our laboratories: MWRg30 (anti-GPIIb/IIIa, IgG1), JON1 (anti-GPIIb/IIIa, IgG2b), EDL1 (anti-GPIIb/IIIa, IgG2a).

Platelet preparation and counting

Mice were bled under ether anesthesia from the retro-orbital plexus. Blood was collected in a tube containing 10% (vol/vol) 0.1 mol/L sodium citrate or 7.5 U/mL heparin, and platelet-rich plasma was obtained by centrifugation at 300g for 10 minutes at room temperature (RT). The platelets were washed twice with phosphate-buffered saline (PBS) by centrifugation at 1300g for 10 minutes and were used immediately. Isolated platelets did not show any signs of activation as shown by flow cytometry (staining for P-selectin and surface-expressed fibrinogen). For determination of platelet counts, blood (20 μ L) was obtained from the retro-orbital plexus of anesthetized mice using siliconized microcapillaries and immediately diluted 1:100 in Unopost kits (Beckon Dickinson, Heidelberg, Germany). The diluted blood sample was allowed to settle for 20 minutes in an improved Neubauer Hemocytometer (Superior, Rad Mergentheim, Germany), and platelets were counted under a phase-contrast microscope at $\times 400$ magnification.

Production of monoclonal antibodies

Female Wistar rats, 6 to 8 weeks of age, were immunized repeatedly with mouse platelets or with purified antigens. The rat spleen cells were then fused with mouse myeloma cells (Ag8.653), and hybridomas were selected in HAT medium. Hybridomas secreting mAbs directed against platelet receptors were identified by flow cytometry. Briefly, a 1:1 mixture of resting and thrombin-activated platelets (10^6) was incubated with 100 μ L supernatant for 30 minutes at RT, washed with PBS (1300g, 10 minutes) and stained with FITC-labeled rabbit anti-rat Ig (DAKO) for 15 minutes. Samples were analyzed on a FACScan (Beckon Dickinson) in the set-up mode. Platelets were gated by FSC/SSC characteristics. Positive hybridomas were subcloned twice before large-scale production. Monoclonal antibodies were produced and purified according to standard methods. Isotype sublasses were determined by enzyme-linked immunosorbent assay (ELISA) with alkaline phosphatase (AP)-conjugated isotype-specific antibodies (Pharmingen): p0p 1, IgG2a; p0p 2, IgG1; p0p 3, IgG2a; p0p 4, IgG2b; p0p 5, IgG1.

Modification of antibodies

Affinity-purified antibodies were fluoresceinated to a fluorescein-protein ratio of approximately 3:1 by standard methods with FITC (Sigma) and separated from free FITC by gel filtration on a PD-10 column (Pharmacia, Uppsala, Sweden). HRP conjugation of mAbs was performed with a labeling kit (Boehringer Mannheim, Mannheim, Germany). F(ab)₂ fragments of p0p 3 and p0p 4 were generated by 24-hour incubation of 10 mg/mL mAb with immobilized pepsin (Pierce). Purity of the F(ab)₂ fragments was checked by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Immunoprecipitation and immunoblotting

Immunoprecipitation was performed as described previously.¹⁴ Briefly, 10^8 washed platelets were surface labeled with EZ-Link sulfo-NHS-LC-biotin (Pierce; 100 μ g/mL in PBS) and subsequently solubilized in 1 mL lysis buffer (Tris-buffered saline containing 20 mmol/L Tris/HCl, pH 8, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 2 μ g/mL aprotinin, 0.5 μ g/mL leupeptin, and 0.5% Nonidet P-40, all from Boehringer Mannheim). Cell debris was removed by centrifugation (15 000g, 10 minutes). After preclearing (8 hours), 10 μ g mAb was added together with 25 μ L protein G-Sepharose (Pharmacia), and precipitation took place overnight at 4°C. Samples were separated on 9% to 15% gradient SDS-PAGE along with a molecular weight marker and were transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with streptavidin-HRP (1 μ g/mL) for 1 hour after blocking. After extensive washing, biotinylated proteins were visualized by chemiluminescence (ECL; Amersham).

For immunoblotting, platelets were not surface labeled. After lysis, whole-cell extracts were run on an SDS-PAGE gel and transferred to a PVDF membrane. The membrane was first incubated with 5 μ g/mL FITC-labeled p0p 5 followed by rabbit anti-FITC-horseradish peroxidase (1 μ g/mL). Proteins were visualized by ECL. Immunoprecipitation of GC from 1 mL of 1:10 diluted (PBS) mouse plasma was performed overnight at 4°C.

Flow cytometry

Freshly isolated platelets were washed twice with PBS and then resuspended in platelet buffer (20 mmol/L Tris HCl, pH 7, 0.9% NaCl, 1 mmol/L CaCl₂, and 1 mmol/L MgCl₂) at a concentration of $4 \times 10^4/\mu$ L. Samples containing 25 μ L of this dilution were stimulated with agonists for 10 minutes at RT, followed by the addition of saturating amounts of fluorophore-labeled antibodies or vice versa. After 15 minutes of incubation at RT, the samples were analyzed on a FACScan. PMA (50 ng/mL; Sigma) or thrombin (0.2 U/mL; Boehringer Mannheim) was used as agonists. For preincubation experiments, platelets were incubated with unlabeled mAbs for 15 minutes followed by the addition of saturating amounts of fluorophore-labeled antibodies. For analysis of ristocetin-induced platelet activation, platelets were incubated with 1.5 mg/mL ristocetin in the presence of 1 U/mL apyrase (grade III; Sigma) for 10 minutes at RT.

Immunohistochemistry

Acetone-fixed cryosections (6 μ m) were blocked (5% normal goat serum, 5 mg/mL bovine serum albumin in PBS) for 30 minutes at RT. Primary mAbs were added at a final concentration of 2 μ g/mL. After 90 minutes, the sections were washed 3 times with PBS and subsequently incubated with the adequate HRP-labeled secondary antibodies at a final concentration of 2 μ g/mL for 60 minutes at RT. The ABC substrate was added after 3 washing steps, and the sections were then counterstained with hematoxylin.

Lipopolysaccharide treatment

Mice were injected with the indicated amounts of LPS (in 0.5 mL sterile PBS) intraperitoneally.

Sequencing

The antigen of 5×10^{10} unbiotinylated platelets was immunoprecipitated with p0p 3. After electrophoresis on SDS-PAGE and transfer to PVDF membrane, the

150-kD band was cut out and enzymatically deblocked with pyroglutamate aminopeptidase (sequencing grade; Boehringer Mannheim) as described.²⁵ The deblocked protein was subjected to an Applied Biosystems (Foster City, CA) protein sequencer (model 494) with an online PTH-analyzer.

Glycocalcin ELISA

Microtiter plates were coated with 15 µg/mL p0p 3 in coating buffer (50 mmol/L NaHCO₃, pH 9) overnight at 4°C. After blocking, serial dilutions of plasma or platelet supernatants were added to duplicate wells (1 hour, 37°C). Plates were washed and subsequently incubated with HRP-conjugated p0p 4 (5 µg/mL, 1 hour, 37°C). After extensive washing, TMB was added to each well, and the reaction was stopped by the addition of 2 N H₂SO₄ after 10 to 15 minutes. Absorbance at 450 nm was recorded on a Multiskan MCC/340 (Labsystems, Lugano, Switzerland).

Glycocalcin standard

The copy number of GPIIb on mouse platelets was estimated by flow cytometry by comparing FITC signals obtained with R-phycoerythrin (PE) conjugated p0p 3-5 on mouse platelets and a PE-conjugated antihuman GPIIb mAb on human platelets at identical instrument settings. The signal intensities obtained were in a similar range. Thus, the number of GPIIb molecules on human and mouse platelets was assumed to be similar (approximately 25 000). Washed platelets (5×10^5 /mouse) from 10 healthy mice (5 NMRI, 5 Balb/c) were suspended in each 500 µL PBS (10^6 platelets/mL) and activated with PMA (50 ng/mL) for 20 minutes at RT. Efficiency of GC shedding in each sample was determined by flow cytometry ($75.6\% \pm 3.4\%$). Platelets and debris were removed by centrifugation (15 000g, 15 minutes). The supernatants were tested in the GC-ELISA and were found to contain virtually identical amounts of GC. Based on the estimated copy number of 25 000/platelet GPIIb, it was calculated that approximately 18 750 GC molecules had been shed from each platelet. Therefore, the supernatants were assumed to contain approximately 1.875×10^{10} GC molecules per milliliter ($18 750 \times 10^6$). Based on a molecular weight of 130 kD of GC, a GC concentration of 23.8 µg/mL in the pooled supernatant was calculated. The supernatant was diluted 1:100 in PBS, and aliquots were snap frozen in liquid nitrogen and stored at -70°C. Serial dilutions of this standard were used as a control of known GC concentration in each experiment. The GC concentration of the standard was defined as 1 arbitrary unit.

Results

p0p mAbs are directed against mouse GPIIb-IX

A series of novel mAbs recognizing a highly expressed membrane glycoprotein complex on mouse platelets was generated. Five of these mAbs (p0p 1-5) were used to characterize the recognized antigen. As shown in Figure 1a, p0p 1-5 precipitated proteins of identical molecular weights (150 and 25 kD under reducing conditions) from resting surface-biotinylated mouse platelets. Although the 150-kD band was precipitated in comparable amounts by all mAbs, different quantities of the 25-kD chain were observed. We used p0p 5 for Western blot analysis of the immunoprecipitates, which demonstrated that the 150-kD proteins precipitated by the p0p mAbs were identical (Figure 1b). N-terminal amino acid sequencing of the enzymatically deblocked protein (see "Materials and Methods") identified the 150-kD protein as mouse GPIIb²⁶ [H(T)(X)(S)ISKVTSLLEV]. Flow cytometric experiments demonstrated that p0p 1-5 recognized nonoverlapping epitopes (not shown). GPIIb and GPIIb/IIIa are expressed in comparable amounts on the surface of resting mouse platelets as determined flow cytometrically by comparing fluorescence intensities obtained with the p0p mAbs and anti-GPIIb/IIIa mAbs (JON1, MWReg30).

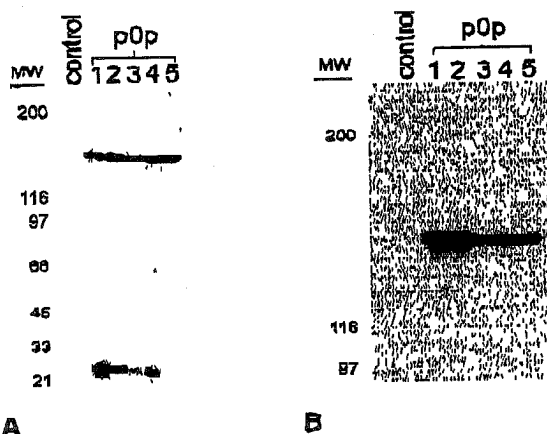


Figure 1. p0p mAbs are directed against mouse GPIIb-IX. (A) Immunoprecipitation from surface-biotinylated resting platelets by p0p 1-5. NP-40 lysates were incubated with nonimmune rat IgG1 (control) or p0p 1-5, followed by protein G-Sepharose. Proteins were separated by 9% to 15% gradient SDS-PAGE under reducing conditions, transferred to a PVDF membrane, and detected by streptavidin-HRP and ECL. (B) Unlabeled platelet proteins were immunoprecipitated with nonimmune rat IgG1 (control) or p0p 1-5, followed by SDS-PAGE and immunoblotting with FITC-labeled p0p 5. Bound p0p 5 was detected by HRP-labeled rabbit anti-FITC.

Shedding of GPIIb is the dominant mechanism of GPIIb-IX down-regulation on activation with thrombin or PMA

As shown in Figure 2a, activation of platelets with thrombin or PMA resulted in decreased binding of the p0p mAbs to the platelet surface, whereas signals for GPIIb/IIIa (JON1) and P-selectin (not shown) significantly increased. Although staining with p0p 3-5 was almost completely abolished on activation, p0p 1,2 signals were just slightly decreased. To discriminate between receptor internalization and proteolytic cleavage, platelets were first incubated with fluorophore-labeled mAbs and activated with thrombin or PMA after 15 minutes (Figure 2b). Again, the results obtained with p0p 1,2 differed significantly from those obtained with p0p 3-5. FITC intensities of p0p 1,2 were virtually unaffected by both forms of activation under these conditions, indicating that the respective epitopes had been internalized. In contrast, fluorescence signals of p0p 3-5 decreased significantly on activation with thrombin or PMA, suggesting that the epitopes recognized by these mAbs had been cleaved from the platelet surface to approximately 47% and 73%, respectively. Immunoprecipitation studies demonstrated that p0p 3-5 recognized a soluble 130-kD fragment of the receptor (GC) in the supernatant of platelets activated with PMA (Figure 2c) or thrombin (not shown), whereas p0p 1,2 and control IgG1 did not. We concluded that p0p 1,2 either bound to GPIX or epitopes on GPIIb/β distinct from the GC portion. We were unable to localize the binding epitopes of p0p 1,2 on either GPIX, GPIIb, or GPIIbβ because separation of the peptide chains by different approaches always abrogated binding of the mAbs. However, because binding of p0p 1,2 was unaffected by proteolytic cleavage of GPIIb, we expected these 2 mAbs to precipitate the untruncated rest of this polypeptide chain (t-GPIIb) retained in the membrane after GC shedding (approximately 20 kD). Because this fragment is disulfide linked to GPIIbβ (25 kD), an approximately 45-kD band had to be detectable under nonreducing conditions. Both the 20-kD band (red) and the 45-kD band (nonred) were identified in the immunoprecipitates of p0p 1,2 but not of p0p 3 (Figure 2d) or p0p 4,5 (not shown).

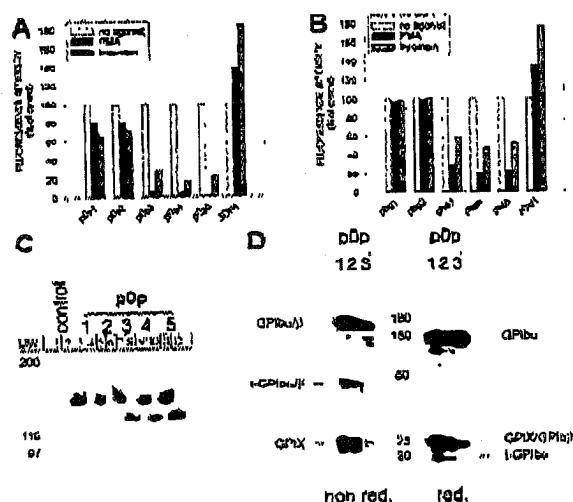


Figure 2. Effect of α -thrombin and PMA on pOp 1-5 binding to mouse platelets. Platelets were first incubated with 0.2 U/mL α -thrombin or 50 ng/mL PMA for 10 minutes at RT and subsequently stained with FITC-labeled mAbs for 15 minutes at RT (A) or vice versa (B). Samples were analyzed on a FACScan. The results shown are representative of 6 individual experiments. (C) Immunoprecipitation with pOp 1-5 and control IgG1 from NP-40 lysates of surface-biotinylated resting platelets (lanes 1, 3, 5, 7, 9, 11) or supernatant of PMA-activated surface-biotinylated platelets (lanes 2, 4, 6, 8, 10, 12; 10 minutes, at 15 000g). Proteins were separated by 9% to 15% SDS-PAGE under reducing conditions, transferred to a PVDF-membrane, and detected by streptavidin-HRP/ECL. (D) Detection of GPIb and the membrane-anchored truncated 20-kd remainder of GPIb (α -GPIb). Surface-biotinylated platelets were stimulated with PMA (50 ng/mL) for 10 minutes at RT, washed twice, and lysed with NP-40. Immunoprecipitates of pOp 1, pOp 2, and pOp 3 (control) were separated under reducing and nonreducing conditions and blotted onto a PVDF-membrane, and biotinylated proteins were detected by streptavidin-HRP/ECL.

Mouse plasma contains approximately 25 μ g/mL glycoalbumin

We performed immunoprecipitation with pOp 1-5 and JON1 (control) from normal mouse plasma and tested the immunoprecipitates for the presence of GC by Western blot analysis with pOp 5. As shown in Figure 3a, pOp 3-5, but not pOp 1,2 or JON1, had precipitated significant amounts of GC. For better quantification of GC in mouse plasma, we established an ELISA system using pOp 3 as capture and HRP-conjugated pOp 4 as detection antibody. Serial dilutions of supernatants from activated (PMA or thrombin) or resting platelets, normal mouse plasma, and a standard of known GC concentration ("Material and methods") were tested (Figure 3b). Results showed that normal mouse plasma contains approximately 25 μ g/mL GC and that a similar amount was shed from 10⁶/mL PMA-activated platelets (physiological count). Therefore, the platelet-GPIb:plasma GC ratio is approximately 1:1 in mouse blood, which was confirmed by a 2-fold increase of GC in platelet-rich plasma on PMA-induced activation. Plasma GC concentrations did not differ significantly between individual mice tested (\pm 5.8%; n = 30; different mouse strains).

Mouse GPIb contains 3 different cleavage sites for platelet-derived proteases

Flow cytometric studies demonstrated that incubation of washed mouse platelets at RT resulted in time-dependent proteolytic degradation of GPIb. Binding of pOp 1,2 remained virtually unaffected for 6 hours, whereas the epitopes recognized by pOp 5

and, to a greater extent pOp 3,4, were down-regulated, indicating progressive shedding of GPIb rather than internalization of the receptor complex (not shown). We took advantage of this observation, incubated freshly isolated surface-biotinylated platelets for 6 hours at RT, and subsequently generated an NP-40 lysate containing the solubilized membrane proteins and all proteolytically cleaved fragments and release products. This lysate was used for immunoprecipitation with the pOp mAbs. Three different band patterns were found on the blot (Figure 4). pOp 1 (lane 2) and pOp 2 (not shown) had precipitated identical bands. Based on these results, we were able to identify 3 different cleavage sites on GPIb, defining fragment pairs of 20 + 130 kd, 90 + 60 kd, and 105 + 45 kd, respectively. pOp 3,4 precipitated the 45-kd band (fragment 1a), whereas pOp 1, 5 precipitated the 105-kd band (fragment 1b). Based on the finding that pOp 1 precipitates the membrane-anchored fragment (see Figure 2d), we concluded that fragment 1a represents the cleaved N-terminal 45-kd fragment of GPIb, whereas fragment 1b is the truncated 105-kd remainder of GPIb retained in the membrane. Therefore, the binding epitopes of pOp 3,4 are located on the N-terminal 45-kd portion of the receptor known to contain the binding sites for vWF and thrombin.⁷⁻⁹ Furthermore, pOp 3-5 precipitated the 60-kd band (fragment 2a), whereas the 90-kd band (fragment 2b) was exclusively recognized by pOp 1 (and pOp 2). The latter therefore represented the membrane-anchored truncated remainder of GPIb. We concluded that pOp 5 binds to an epitope on the N-terminal 60-kd portion of GPIb located between cleavage sites 1 and 2. The 130-kd band (fragment 3a), recognized by pOp 3-5, was identified as GC, whereas the 20-kd band (fragment 3b) exclusively recognized by pOp 1,2 represented the corresponding membrane-anchored truncated form of GPIb (α -GPIb).

Occupancy of GPIb induces irreversible aggregate formation in vitro and rapid thrombocytopenia in vivo

Standard aggregometry demonstrated that none of the pOp mAbs (at concentrations of 10, 30, or 100 μ g/mL) interfered with aggregation induced by adenosine diphosphate, collagen, PMA, or ristocetin (not shown). We observed, however, that ristocetin (at concentrations greater than 1 mg/mL) did not induce passive

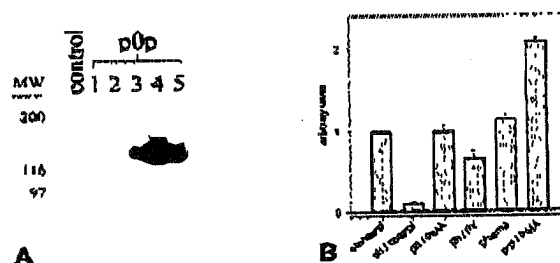


Figure 3. Detection and quantification of glycoalbumin in mouse plasma. Blood (50 μ L) was collected in 200 μ L heparinized PBS and diluted 1:10 with PBS. Cells and microparticles were removed by centrifugation. (A) Immunoprecipitation from normal mouse plasma with pOp 1-5 and a nonimmune IgG1 (control), followed by immunoblotting with pOp 5-FITC. Bound pOp 5 was detected by HRP-labeled rabbit anti-FITC/ECL. (B) Detection and quantification of glycoalbumin in mouse plasma and the supernatant of mouse platelets using a sandwich-type ELISA (pOp 3,4-HRP). Washed platelets (10⁶/mL) or platelet-rich plasma were activated with either PMA (50 ng/mL) or thrombin (100 U/mL) or were incubated without agonist (control) for 15 minutes at RT. Supernatants were prepared by centrifugation at 15 000g for 10 minutes and were tested along with normal mouse plasma and a standard of known GC concentration (23.6 μ g/mL) in serial dilutions. See "Materials and methods" for details.

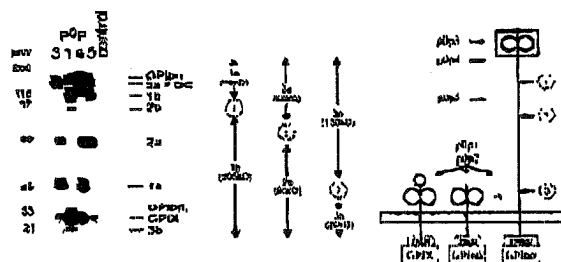


Figure 4. Immunoprecipitation of 6 different proteolytic fragments of GPIIb/IIIa. Surface-biotinylated mouse platelets were incubated for 6 hours at RT and lysed directly. Immunoprecipitation was performed with pOp 1,3,4,5 and control IgG1. Proteins were separated by 9% to 15% SDS-PAGE under reducing conditions and were detected by streptavidin-HRP/ECL. (right) Schematic drawing of the murine GPIIb/IIIa complex. (arrows) Proposed cleavage sites (1, 2, 3). (left, arrows) Assumed binding sites of pOp 1-5. (middle) Schematic drawing of fragment pairs shown on the blot.

agglutination (as described for human platelets) but did induce active aggregation of mouse platelets as evidenced by rapid surface expression of P-selectin, fibrinogen, and vWF in the presence of this antibiotic (Figure 5).

Although no inhibitory effect of the pOp mAbs on platelet aggregation could be detected, direct platelet activation by all mAbs directed against the OC portion of GPIIb/IIIa was obvious. Addition of (10-100 μ g/mL) pOp 3-5 or F(ab)₂-fragments of pOp 3,4, but not pOp 1,2 or control IgG, to platelet-rich plasma under stirring conditions (1000 rpm, 37°C) induced the formation of microaggregates of 3 to 5 platelets (not shown). Platelet activation induced by pOp 3-5 became more evident after mild centrifugation of the samples (1300g, 5 minutes, RT).

After resuspension, large aggregates were found, whereas the single platelet count was drastically reduced (Figure 6a). Flow cytometric analysis of the aggregates demonstrated no increased surface expression of P-selectin, vWF, or fibrinogen (not shown). In correlation with platelet aggregating effects in vitro, we found rapid and irreversible platelet depletion on the injection of 25 μ g per mouse pOp 3-5 or F(ab)₂-fragments of pOp 3,4, whereas thrombocytopenia induced by pOp 1,2 or EDL1 (anti-gpIIb/IIIa) was less effective (Figure 6b).

GPIIb/IIIa is not detectable on endothelial cells under normal or inflammatory conditions

To examine the in vivo protein expression of GPIIb/IIIa, cryosections from the major organs (spleen, lung, liver, kidney, heart, brain, intestine, skin, and thymus) from normal mice ($n = 6$) were stained for GPIIb/IIIa with a 1:1 mixture of pOp 1 and pOp 4 (each at 2 μ g/mL). Antibodies against vWF were used as a control for staining the platelets, megakaryocytes, and endothelial cells.

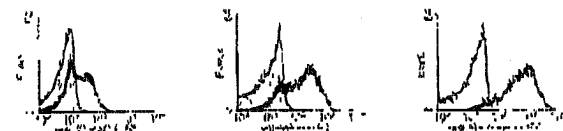


Figure 5. Ristocetin induces activation of mouse platelets. Platelets (10^6) were incubated with 1.5 mg/mL ristocetin in the presence of 1 U/mL apyrase at RT for 10 minutes. Subsequently, FITC-labeled antibodies were added in saturating amounts, and the samples were analyzed on a FACScan after 15 minutes. (shaded area) Staining of resting platelets. (solid lines) Staining of ristocetin-activated platelets.

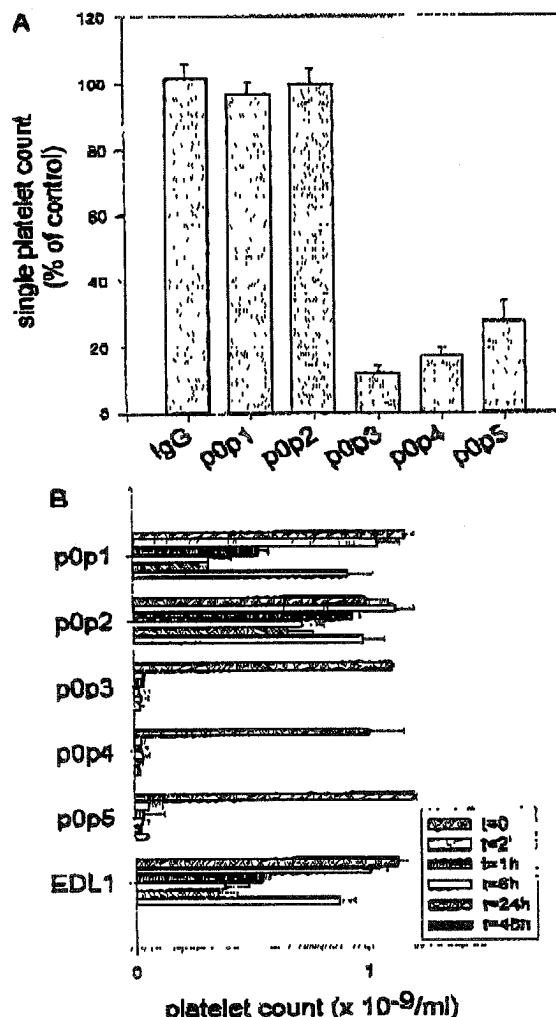
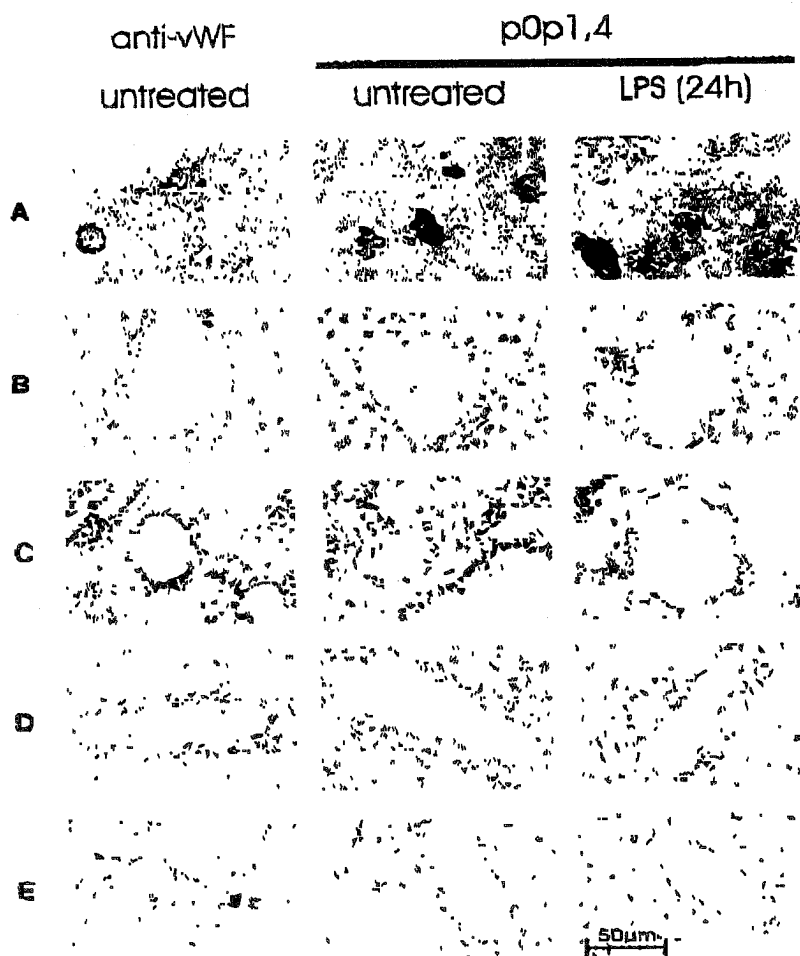


Figure 6. pOp 3-5 induces aggregate formation in vitro and rapid thrombocytopenia in vivo. (A) Washed platelets (2×10^6) were incubated with 10 mg/mL of the indicated mAbs or without antibody (control) for 15 minutes at RT, followed by centrifugation at 1300g for 5 minutes. After resuspension of the pellets by vortexing for 3 seconds, the single platelet count in the samples was determined by flow cytometry. Results are shown as the mean \pm SD for 3 independent experiments. (B) Normal female NMRI mice received 25 μ g purified pOp 1,2,5 or F(ab)₂-fragments of pOp 3,4 intravenously in 200 μ L sterile PBS. The anti-GPIIb/IIIa mAb EDL1 was used as a control. Platelet counts were determined at the indicated times using an improved Neubauer hemocytometer. Injection of a nonimmune IgG1 had no significant effect on the platelet count (not shown). Intact pOp 3,4 induced comparable thrombocytopenia as the F(ab)₂-fragments (not shown). Results of platelet count are shown as the mean \pm SD for groups of 3 mice. The experiment was repeated twice with comparable results.

pOp1,4, and anti-vWF specifically stained megakaryocytes and platelets in the red pulp of the spleen (Figure 7). In the lungs, only platelets but not endothelial or other cells were stained with pOp 1 and pOp 4, whereas anti-vWF stained platelets and endothelial cells. In the liver, kidney, and heart, few platelets were detectable with pOp 1, pOp 4, and anti-vWF. As in the lungs, specific staining

Figure 7. Immunohistochemical detection of GPIb-IX. Acetone-fixed frozen sections from normal (untreated) mice and mice 24 hours after injection of 20 mg/kg LPS were stained for GPIb-IX with p0p 1,4. As a positive control for platelet/megakaryocytic and endothelial expression, sections from normal mice were stained for vWF (left). Representative sections of (A) spleen, (B) liver, (C) lung, (D) kidney, and (E) heart are shown.



of endothelial cells was only detectable with anti-vWF. The same result was obtained with brain, intestine, skin, and thymus (not shown), in which virtually no platelets were detected. The staining pattern obtained with p0p 1, p0p 4, and JON1 (anti-GPIIb/IIIa, not shown) was identical in all organs examined. Thus, no expression of GPIb-IX on cells other than platelets/megakaryocytes was detectable in any organ of normal healthy mice. To test the hypothesis that GPIb-IX might be expressed on endothelial cells in response to inflammatory stimuli,^{10,29,30} we treated mice with bacterial LPS (20 mg/kg, $n = 20$). It is well documented that such treatment induces the production of a variety of cytokines, resulting in systemic inflammatory responses in mice.³⁷ Significant thrombocytopenia (platelet count, $61.4\% \pm 4.9\%$ of normal) and hypothermia ($-3.3^\circ\text{C} \pm 0.6^\circ\text{C}$) developed in all mice within 12 hours. Organs from 5 mice were sampled after 6, 12, 24, or 48 hours, and cryosections were stained with p0p 1, p0p 4, anti-vWF, or JON1. Although all antibodies stained platelets and megakaryocytes, specific staining of endothelial cells in these organs was only observed with anti-vWF. A significantly increased number of platelets in the livers of LPS-treated mice was detected with p0p 1 and p0p 4 (and JON1), whereas staining of platelets in the lungs

and platelets/megakaryocytes in the spleens was apparently unchanged compared with that in the control mice. As in the control mice, kidney, heart, skin, intestine, brain, and thymus appeared virtually free of platelets (the latter 4 organs are not shown).

Discussion

In the current study we investigated the structural and functional properties of mouse GPIb-IX and systematically examined the *in vivo* expression of the receptor on a cellular level for the first time. For our studies we used 5 newly developed monoclonal antibodies that recognized different, nonoverlapping epitopes on the complex. Immunoprecipitation and Western blot analysis showed that the individual components of the complex had apparent molecular weights similar to those of human homologs: GPIb α (approximately 150 kD), GPIb β (approximately 25 kD), and GPIX (approximately 25 kD) (Figures 1, 2). Using p0p 1-5 for flow cytometric and biochemical analyses, we were able to examine and quantify the regulation of different epitopes on mouse GPIb-IX under various experimental conditions. In our experiments, we found that throm-

bin-induced activation of mouse platelets resulted in approximately 47% shedding of GPIb α (GC) and only approximately 31% internalization of the GPIb-IX complex. In contrast, thrombin has been reported to induce internalization of GPIb-IX complexes from the platelet surface to the surface-connected canalicular system without evidence for receptor shedding on human platelets.²⁰⁻²² though this finding is not commonly accepted.^{23,24} Therefore, it seems likely that mouse GPIb α is more susceptible to proteolytic cleavage during platelet activation. This hypothesis may be supported by the detection of at least 10-fold higher GC concentrations in mouse plasma than in human plasma (approximately 25 $\mu\text{g/mL}$ versus approximately 2 $\mu\text{g/mL}$).²⁵ Certainly, the higher platelet counts in mice ($1.0\text{--}1.2 \times 10^6/\mu\text{L}$ versus $0.2\text{--}0.4 \times 10^6/\mu\text{L}$) also contribute to the marked difference in plasma GC concentrations between the 2 species.

Although GC is the only known fragment of human GPIb α generated by platelet-derived proteases, an additional N-terminal 45-kd fragment can be generated experimentally by trypsin digestion.⁸ This 45-kd fragment, isolated by tryptic digestion or expressed by recombinant DNA methods, contains the binding sites for vWF and thrombin and essentially mimics the functional properties of GPIb-IX-V as a soluble receptor.⁷⁻⁹ In our immunoprecipitation experiments with washed platelets, we identified 3 different soluble fragments (GC, 60 kd and 45 kd) and the 3 corresponding membrane-anchored truncated forms of GPIb α (20 kd, 90 kd, and 105 kd), demonstrating that these fragments must have been generated by platelet-derived proteases (Figure 4). To our knowledge, this is the first report describing the proteolytic generation of both the 45-kd and the 60-kd N-terminal fragment of GPIb α by platelet-derived proteases. Based on our data, we propose 3 cleavage sites on murine GPIb α (Figure 4), suggesting complex regulation mechanisms of GPIb-IX function in vivo. In contrast, in vitro activation of platelets with thrombin or PMA only resulted in proteolytic generation of GC but not of the 45-kd and 60-kd fragments of GPIb α (not shown). Most information on GPIb-IX-V regulation in humans is derived from studies using exogenous platelet-activating stimuli. This may explain why GC is the only known proteolytic fragment of GPIb α cleaved from activated human platelets.

The GPIb α -vWF interaction has become a potentially interesting target for antithrombotic therapies,³⁹ leading to the development of strategies for receptor or ligand blockage in vivo. Although mAbs against the A1-domain of vWF efficiently blocked GPIb α -vWF interaction in vivo,³⁹ there are conflicting reports about the in vivo effects of anti-GPIb α mAbs. Becker et al⁴⁰ reported that F(ab)₂-fragments of an anti-GPIb α mAb inhibited GPIb-vWF interactions in vivo and ex vivo without significant effects on platelet counts in guinea pigs. In contrast, a more recent study performed in baboons showed that the injection of anti-GPIb α -F(ab)₂-fragments immediately resulted in profound irreversible thrombocytopenia.⁴¹ We observed similar effects in the mouse. All mAbs or F(ab)₂-fragments against the GC portion of GPIb α , irrespective of their exact binding epitope, induced aggregate formation in vitro and a greater than 95% drop of platelet count within 2 minutes in vivo (Figure 6). Although the mechanisms underlying this cytotoxic effect could not be identified in the current study, it seems possible that attempts to block certain epitopes on GPIb with modified antibodies in vivo may generally result in thrombocytopenia. In contrast to the inhibition of GPIbIIIa function,⁴² in vivo blockage of certain epitopes on GPIb may, therefore, not be a promising antithrombotic strategy.

Monoclonal antibodies directed against human GPIb α have been reported to inhibit ristocetin-induced platelet agglutination and to interfere with collagen-induced platelet aggregation.⁴³ Our aggregometric studies with p0p 1-5, however, showed that none of the mAbs had significant influence on aggregation induced by adenosine diphosphate, collagen, and PMA (not shown). Furthermore, p0p 1-5 had no effect on ristocetin-induced platelet aggregation, which was always associated with classical platelet activation. Concentrations greater than 1 mg/mL ristocetin induced surface expression of P-selectin, fibrinogen, and endogenous vWF, as determined by flow cytometry (Figure 5). The direct activation of mouse platelets by ristocetin contrasts its passive agglutination of human platelets.¹⁴ Thus, ristocetin may not be suited for studies on GPIb α -vWF interactions in the mouse system.

The expression of GPIb-IX on cells of nonmegakaryocytic origin (particularly endothelial cells) has been controversial. Although the importance of the complex in normal megakaryocyte and platelet physiology is clear and well documented, some authors have speculated on a second, unrelated role of GPIb or the GPIb-IX-V complex.²⁹⁻³¹ This hypothesis is based on the observation that cultured human endothelial and smooth muscle cells express the individual subunits of the complex.^{31,44-46} Furthermore, endothelial GPIb α has been proposed to be involved in platelet-endothelial cell interactions under inflammatory conditions.^{29,30,47} On the other hand, these data are not without debate because the findings could not be reproduced by others.³⁹ Recently, Fujita et al⁴⁸ made the first attempt to examine the in vivo expression of GPIb α in the mouse. The authors reported consistent and reproducible GPIb α gene activity in nonhematopoietic organs, including lung and heart, and they speculated on GPIb α expression on cells other than platelets/megakaryocytes. Although GPIb-IX-V can be detected immunohistochemically on cultured human endothelial cells,²⁹⁻³¹ no systematic studies on the in vivo expression of the receptor on a cellular level have been performed to date. We examined GPIb-IX expression under normal and inflammatory conditions immunohistochemically for the first time and found specific staining in the major organs of the mouse only on platelets and megakaryocytes, but not on endothelial cells (Figure 7). Comparison between JON1 (anti-GPIbIIIa) and p0p 1,4 (anti-GPIb-IX)-stained sections demonstrated identical staining patterns in all organs examined. In contrast, antibodies against vWF clearly stained platelets/megakaryocytes and endothelial cells. Although the sensitivity of immunohistochemical techniques is limited, our studies did not support the hypothesis that GPIb-IX is expressed on endothelial cells in vivo.

In conclusion, the results presented in this article indicate that mouse GPIb-IX was exclusively expressed on platelets and megakaryocytes and, despite some differences, shared many structural and functional properties with the human receptor. The p0p mAbs proved powerful tools, and they may be helpful for further studies on the function and regulation of the GPIb-IX complex. The availability of transgenic and knockout strains predestinates the mouse system for such investigations.

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REVIEW ARTICLE

Therapeutic approaches in arterial thrombosis

D. R. PHILLIPS, P. B. CONLEY, U. SINHA and P. ANDRE
Portola Pharmaceuticals, Inc., San Francisco, CA, USA

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Summary. The current standard of care for the treatment of arterial thrombosis includes anticoagulants and three classes of antiplatelet agents – aspirin, thienopyridines and glycoprotein IIb-IIIa antagonists. Although these drugs have had a significant impact on morbidity and mortality in several patient populations, up to 15% of the high risk patients with acute coronary syndrome continue to suffer from ischemic events. This problem may occur, in part, because the platelets in many patients are non-responsive to aspirin and clopidogrel. Murine models now indicate that platelets are not only responsible for arterial occlusion, they are also involved in the progression of atherosclerotic disease. New opportunities have emerged identifying potential targets and strategies for drug discovery suited to address these deficiencies by more effectively modulating platelet adhesion, thrombus growth, thrombus stability and the pro-inflammatory activity of platelets. In addition, a growing need has emerged for the development of bedside devices capable of bringing personalized medicine to patients being treated with antithrombotic drugs in order to measure the pharmacodynamic activities of new therapies, to assess the activities achieved by combined antithrombotic therapy, and to identify patients that fail to respond.

Keywords: anticoagulant, antiplatelet, atherosclerosis, platelet, platelet monitoring, thrombosis.

Introduction

Arterial thrombosis is the result of sequential events involving platelet adhesion, activation and subsequent aggregation that can lead to vascular occlusion, perhaps the primary pathological complication of advanced atherosclerotic lesions. Recent advances in the field of thrombosis suggest that the second pathological consequence of platelet adhesion and activation may be as consequential as the immediate ischemia induced by arterial thrombosis as platelets are a primary source of several inflammatory proteins known to be involved in the progression of atherosclerotic disease including RANTES, sCD40L, PDGF and transforming growth factor- β (TGF- β). These considerations suggest that therapeutic targeting of platelets

has two objectives: first, prevention of vessel occlusion; second, inhibition of the platelet contribution to lesion progression.

The pharmaceutical industry has made important inroads into the development of drugs for the treatment of the thrombotic complications of atherosclerosis. In one example, the occlusive, ischemic consequences of acute myocardial infarction (MI) have been addressed, by thrombolytics to lyse the thrombus, and more recently by interventional strategies to mechanically remove or dislodge the thrombus and to maintain artery patency with stents coated with agents such as rapamycin or paclitaxel to reduce the incidence of restenosis. In yet another example, antagonists against platelet receptors such as glycoprotein (GP) IIb-IIIa and P2Y₁₂ have been developed, joining aspirin, a Cox-1 inhibitor as the primary antithrombotic drugs. However, despite these advances in antithrombotic therapies and the widespread use of statins to reduce cholesterol and CRP levels, the incidence of atherosclerosis continues to rise, as do the ischemic consequences of atherosclerosis including MI and stroke. An added complication is type 2 diabetes which is an independent risk factor for cardiovascular disease (CVD). A recent analysis of the Framingham Heart Study showed that even though management of risk factors such as blood pressure and cholesterol has improved significantly in the total patient population, the presence of diabetes significantly reduced the overall benefits [1]. While it may be possible to address these pathologies by more aggressive health management, and by more optimal application of existing therapies, clearly, truly effective treatment of the thrombotic consequences of atherosclerosis requires not only the development of drugs to be used as primary care on a chronic basis to prevent thrombosis and its ischemic complications but also to block the contribution of platelet-induced inflammation in the progression of atherosclerotic disease. Reviewed below is the mechanism of action, clinical successes and limitations of the four drug classes currently used to prevent arterial thrombosis; aspirin, P2Y₁₂ inhibitors, GP IIb-IIIa antagonists and anticoagulants. Therapeutic opportunities afforded by our current understanding of the mechanisms of arterial thrombosis and the inflammatory activity of platelets are discussed. Finally, recognizing that individuals vary in response to various drugs and that combination antithrombotic therapies has become commonplace, we will highlight the need for improved pharmacodynamic assessment of platelet function.

Correspondence: David R. Phillips, Portola Pharmaceuticals, Inc., 270 E. Grand Ave., South San Francisco, CA 94402, USA.
Tel.: +1 650 246 7505; fax: +1 650 246 7776; e-mail: dphillips@portola.com

Current strategies

Current therapeutic strategies for the treatment of arterial thrombosis are based on the well-known receptor systems summarized in Fig. 1. In this simplified diagram, collagen and/or thrombin are designated as the primary platelet agonists. While either agonist is capable of activating platelets, including the activation of the receptor function of GP IIb-IIIa for the binding of fibrinogen and von Willebrand factor (VWF) to initiate platelet aggregation, stable aggregation of platelets is augmented by two autocrine factors generated upon platelet stimulation: ADP, released from platelet dense bodies, and TXA_2 , generated by the sequential actions of Cox-1 and thromboxane synthase on the arachidonic acid released from membrane phospholipids. Additional aggregation-dependent secondary mediators include sCD40L and Gas6 plus aggregation-induced tyrosine phosphorylation of GP IIIa and activation of secondary aggregation receptors such as SLAMF1, CD84, Eph kinase and the Gas-6 receptors. Even though the signaling reactions induced by the receptor systems for the platelet stimuli summarized in Fig. 1 are diverse, including those coupled by G_q (PAR-1 and TP), G_i ($P2Y_{12}$), Syk (GP VI), Shc and talin (GP IIb-IIIa), drugs that target these receptor systems have been designed either to specifically inhibit the receptors themselves [e.g. GP IIb-IIIa antagonists (eptifibatide, abciximab, tirofiban) and $P2Y_{12}$ inhibitors (clopidogrel, ticlopidine)], to block the generation of the agonists [e.g. the Cox-1 inhibitor aspirin and Factor Xa (FXa) inhibitors [low molecular weight (LMW) heparins]], or to antagonize the agonist itself (e.g. thrombin inhibitors (standard heparin, direct thrombin inhibitors)).

Aspirin

The clinical successes achieved by the current therapies to treat arterial thrombosis have been remarkable. Aspirin was the first

and continues to be the most widely used of these drugs. The trend toward the widespread use of this drug to block arterial thrombosis was first indicated by the findings of the ISIS-2 trial which demonstrated that aspirin reduced mortality from acute MI to a rate that is similar to that of the thrombolytic agent, streptokinase [2]. The data from multiple trials summarized by the Antiplatelets Trialists' Collaboration found a 25% relative risk reduction by aspirin of vascular death, MI or stroke, vs. placebo [3] which led to the widespread adoption of aspirin as standard therapy for primary and secondary prevention of arterial ischemia. This collaboration also reviewed the clinical trials using aspirin to show that low-dose aspirin (75–150 mg daily) is effective for long-term use [4]. While the half-life of aspirin in humans is relatively short (~20 min), its effect persists for the lifetime of an affected platelet in circulation as the drug acetylates Cox-1 at serine-529, located at the active site of the enzyme. Attempts have been made to develop additional drugs that target the thromboxane pathway in platelets including a variety of thromboxane receptor (TP) antagonists, thromboxane A_2 synthase inhibitors, or compounds that combined both functions [5]. Although some of these agents had potent antithrombotic effects in experimental models and preclinical studies, and offered the advantage of inhibiting the TP stimulating activity of prostanoid metabolites in addition to TXA_2 (e.g. isoprostanes, PGH_2), they are not currently used to block arterial ischemia as most were not evaluated in clinically relevant phase III trials [6].

Thienopyridines

The second most widely used of the antiplatelet drugs for chronic use are thienopyridines targeting $P2Y_{12}$. This class of drugs, which includes clopidogrel, and its predecessor ticlopidine, act via irreversible inhibition of the platelet $P2Y_{12}$ receptor. Both are prodrugs, requiring hepatic metabolism by

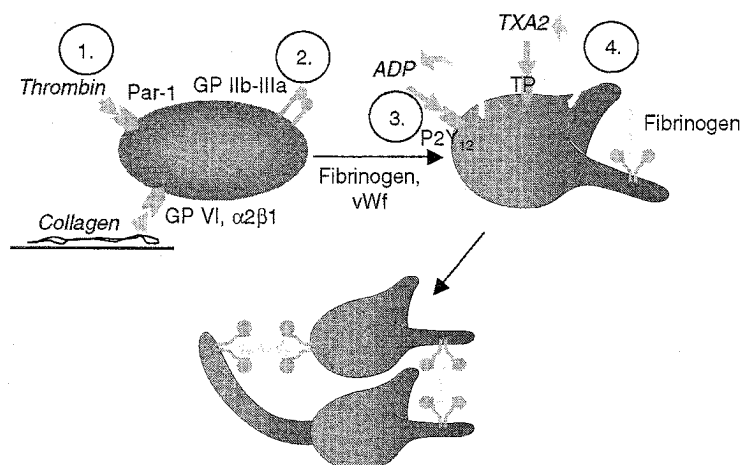


Fig. 1. Current therapeutic strategies for the treatment of arterial thrombosis. The reaction diagram is designed to illustrate the pathways regulated by current therapeutic strategies on unstimulated, discoid platelets (top left), stimulated platelets (top right), and aggregated platelets (bottom). The four antithrombotic drug classes include: (1) Thrombin inhibitors; (2) GP IIb-IIIa antagonists; (3) $P2Y_{12}$ antagonists; and (4) inhibition of TXA_2 production.

cytochrome P450 isoform 3A4 in order to generate the active metabolite, a transient intermediate which covalently modifies and inactivates the receptor. Ticlopidine has been shown to be efficacious in conditions such as claudication, unstable angina, and cerebrovascular disease [7]. However, the incidence of neutropenia associated with ticlopidine led to the development of a second-generation thienopyridine, clopidogrel, with increased potency and fewer side-effects. In the CAPRIE trial [8], clopidogrel was shown to be more efficacious than aspirin, particularly in high-risk patients (diabetics and those with a history of prior revascularization). Subsequently, the CURE study [9] demonstrated that patients with unstable angina or non-ST segment elevation MI received a 20% relative risk reduction if they were randomized to clopidogrel plus aspirin vs. placebo plus aspirin, and the PCI-CURE substudy [10] showed that this benefit extended to patients undergoing percutaneous intervention (PCI). The slow onset of action of thienopyridines, due to their metabolism requirement, has necessitated the administration of a large loading dose (300 mg) prior to acute procedures, such as PCI, as demonstrated in the CREDO trial, where the maximum benefit of clopidogrel administered with aspirin required a loading dose given at least 6 h prior to the procedure. This study also demonstrated a significant 27% reduction in death, MI and stroke from 1-year administration of clopidogrel plus aspirin following PCI, compared to 1-month dosing [11].

GP IIb-IIIa antagonists

The GP IIb-IIIa antagonists are designed to bind to the integrin on unstimulated platelets and on platelets after stimulation. GP IIb-IIIa is an attractive antiplatelet target as it is (i) on the 'final common pathway' mediating platelet aggregation irrespective of the agonist used to induce platelet activation, (ii) platelet-specific, and (iii) responsible for a variety of aggregation-dependent platelet functions including those in coagulation, inflammation, fibrinolysis and vascular cell proliferation. Three GP IIb-IIIa antagonists have been developed: integrilin, a cyclic heptapeptide modeled after the active site of the disintegrin found in the southeast pigmy rattle snake; abciximab, a Fab fragment of a mouse/human chimeric antibody against GP IIb-IIIa; and tirofiban, a synthetic inhibitor of GP IIb-IIIa. All were designed to be infusible i.v. drugs and are therefore only administered to patients in acute settings who have a high risk of experiencing an ischemic event such as those undergoing PCI (with or without stent placement) or those with symptoms resulting from acute coronary syndrome (ACS) [12]. Use of these drugs has shown a remarkable reduction in death and MI for these indications [13–15].

Anticoagulants

ACS patients, whether undergoing an invasive revascularization procedure or not, are treated with aspirin and antithrombin therapy in the form of unfractionated or LMW heparins. Although unfractionated heparin is effective in reducing clinical

events, a narrow therapeutic index makes it a less than optimal antithrombin for this class of patients. Like their parent anticoagulant, i.e. standard heparin, LMW heparins are indirect antithrombins and utilize antithrombin III to mediate inhibition of thrombin and FXa. As LMW heparins have more predictable pharmacokinetics than standard heparin, they are used in a fixed dose manner. Early trials of the LMW heparin enoxaparin in unstable angina and non-Q wave MI patients demonstrated improved efficacy over standard heparin and the drug has emerged as the most commonly used LMW heparin [16,17]. Fondaparinux, a synthetic pentasaccharide, also utilizes the antithrombin III binding region of heparin and has been found to be an appropriate anticoagulant for prevention of deep vein thrombosis in orthopedic surgery [18]. Unlike enoxaparin, which inhibits both thrombin and FXa, fondaparinux acts only as an indirect FXa inhibitor. Venous thromboembolism prevention trials showed that fondaparinux has a superior efficacy profile to its comparator enoxaparin. Ongoing trials of fondaparinux in ACS patients will show if the concept of attaining superior efficacy by inhibition of FXa alone (vs. the combination of FXa and thrombin) can be achieved in arterial settings.

Combination antithrombotic therapy

Arterial thrombosis developed at sites of spontaneously or mechanically disrupted atherosclerotic plaque is triggered by a multitude of highly thrombogenic materials (i.e. fibrillar collagen and tissue factor). It is the result of complex interrelations between coagulation and platelets orchestrated by local rheological conditions. An emerging strategy in the treatment of arterial thrombosis came with the realization that combinations of antithrombotics provide greater therapeutic benefit than are provided by drugs used singly. Accordingly, the combination aspirin-plus-clopidogrel is rapidly becoming the new standard of care for the management of patients with non-ST segment elevation ACS and in patients undergoing a PCI. In support of this trend, the CURE study demonstrated that aspirin-plus-clopidogrel caused a 20% relative risk reduction of vascular death, MI, and stroke compared with aspirin-plus-placebo [9]. The dual antiplatelet therapy (aspirin-plus-clopidogrel) was also more effective and safer than a combination aspirin-plus-warfarin in coronary artery stenting [19,20]. The remarkable efficacy of the dual anti-platelet therapy has prompted the initiation of several clinical trials in indications as diverse as atrial fibrillation, peripheral arterial disease, peripheral arterial bypass surgery, secondary and high-risk primary prevention, acute ST-segment elevation MI and heart failure [21]. Finally, although anticoagulants were routinely used in the development of antiplatelet agents, analysis of these data shows that these combinations often provided a clinical benefit that was greater than anticipated. We and others have used thrombosis models to evaluate synergisms between various pathways. Because TXA₂ and ADP activate different pathways, it was anticipated that combinations of inhibitors of the two pathways would confer a

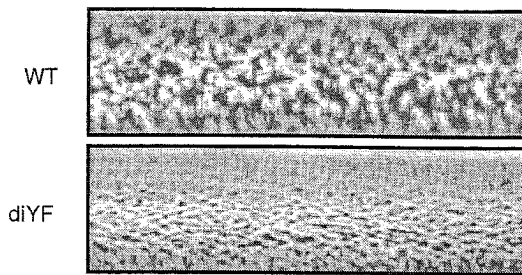


Fig. 2. Thrombosis defect in platelets from the diYF mouse. Unanticoagulated blood from a control mouse (WT) or from a mouse harboring the diYF mutation in GP IIIa (Y747F, Y759F) was perfused for 2.5 min through a chamber coated with type III collagen at 871 s^{-1} and stained. The view is en face.

greater protection against thrombotic events. However, rather than an additive effect, the two drugs used together were synergistic [22,23]. A potent synergism between clopidogrel and anticoagulants [using either a direct thrombin inhibitor (Bivalirudin), a FXa inhibitor or a synthetic LMW heparin] has also been observed (Fig. 2) [23,24]. While these results may have a simple explanation, resulting from the inhibition of fibrin formation by the anticoagulant, where fibrin contributes to thrombus stability under arterial shear rate [25], the emerging data indicate that the anti-thrombotic synergisms may originate from the complementation of the signaling pathways in platelets. Activation of the receptor function of GP IIb-IIIa is optimal when engagement of $G_{12/13}$ or G_q signaling pathways is combined with G_i stimulation [26,27]. This combination occurs when either TXA_2 (TP) receptor ($G_{12/13}$, G_q), protease-activated receptor-1 (PAR-1) ($G_{12/13}$, G_q), and also potentially P2Y_1 (G_q) [28] is allowed to synergize with that of P2Y_{12} (G_i). Note that this model might also explain the synergism between aspirin and clopidogrel. A different explanation may stem from the inhibition of the different PI-3 kinase enzymes present in platelets. For example, P2Y_{12} engagement by ADP stimulates PI-3 kinase- γ whereas the engagement of either $\text{Fc}\gamma\text{RIIA/GPVI}$, PAR-1, TP, or GP IIb-IIIa lead to PI-3 kinase α or β -activation. This could indicate that any combination therapy that would block PI-3 kinase α , β or γ would confer a strong antithrombotic efficacy.

Limitations of current antithrombotics

Even with the remarkable successes that have been achieved with currently available antithrombotics in the prevention of arterial thrombosis, limitations of this class of drugs do exist. It is valuable, therefore, to consider additional strategies currently available to design new drugs that address these limitations. That there is room for improvement is readily apparent from analysis of current trials. For example in SYNERGY, a trial with more than 10 000 high risk non-ST segment elevation ACS patients treated with heparin or LMW heparin, aspirin, and, as determined by the physician, clopidogrel and/or GP IIb-IIIa antagonists, approximately 15% of all patients still

experienced death or non-fatal MI within 30 days of treatment [29]. Except for aspirin, perhaps the biggest limitation of these drugs is that the dose used is less than optimal for the treatment of thrombosis. The drugs were typically titrated in preclinical development studies to arrive at a dose that had a significant inhibition of thrombosis without undue bleeding. While higher doses of the drugs had better antithrombotic activity, this always created a bleeding risk. Although the dose selected required some refinement in subsequent clinical studies, this same strategy was employed.

Aspirin non-responsiveness

While aspirin is used at a dose (e.g. 70–325 mg day⁻¹) that yields near 100% acetylation of Cox-1 in most individuals, it has been recognized for several years that individuals treated with aspirin still experience thrombotic events. The interpretation of this observation has been controversial. One could argue that aspirin is a comparatively 'weak' inhibitor in that it only blocks the production of TXA_2 , an autocrine factor that supplements the activities of the primary platelet agonists. Indeed, aggregation reactions using platelets from individuals taking aspirin appear normal when high concentrations of primary agonists are used. However, it has become clear from recent pharmacodynamic and biochemical studies that platelet responses normally blocked by inhibition of Cox-1 (the target of aspirin in platelets) are still present in some individuals, an observation that has led to the concept of 'aspirin resistance' or 'aspirin non-responders' [30–32]. Studies of platelet aggregation in aspirin-treated CVD patients, both by traditional ADP and arachidonic acid induced aggregation studies and by platelet function analyzers such as PFA100, show that 5–10% of the individuals can be classified as aspirin non-responders [31]. Additional studies demonstrated a threefold higher risk of major cardiovascular adverse events in the same patient population [30]. As the incidence of aspirin non-responders had been shown to be higher in patients who undergo coronary artery bypass graft (CABG), a recent study evaluated the functional and biochemical responses to aspirin on subsequent days following a CABG procedure in a small number of patients [33]. The study demonstrated that platelets from these patients after CABG did not completely respond to aspirin *in vitro*, and that while Cox-1 levels in platelets remained constant 10 days following the procedure, there was a pronounced increase in the level of Cox-2, which is ~170-fold less sensitive to aspirin inhibition, especially at 5 days post-procedure. This may be reflective of the increased platelet turnover following cardiopulmonary bypass, and the increased level of Cox-2 could generate critical amounts of TXA_2 , in spite of aspirin treatment, providing a possible explanation for aspirin non-responsiveness. Other mechanisms proposed as contributing factors to aspirin non-responsiveness include use of NSAIDs, which block acetylation by aspirin [34] and polymorphisms of platelet genes (Cox-1 or GP IIb-IIIa) [35], and non-compliance. However, the answer may be totally unexpected such as the activation of a deacetylase. Clearly, further

studies are needed to better define the underlying mechanisms of this phenomenon. Aspirin use is also contraindicated in a significant population of patients, i.e. those with gastrointestinal bleeding and those with aspirin-induced asthma.

Clopidogrel non-responsiveness

The primary limitation of clopidogrel is that this drug demonstrates weak and somewhat variable inhibition of P2Y₁₂ [36]. Following a 600-mg loading dose of clopidogrel, the extent of inhibition of ADP-induced aggregation (5 μ M ADP) varied from 33% to 78% in healthy individuals, at 6 h post-dosing [37]. This effect is further exaggerated in patients undergoing PCI/stent placement [38,39]. The antithrombotic effect of clopidogrel is likely to be dependent on a number of factors including but not limited to variations in P450s, polymorphisms of the P2Y₁₂ receptor and receptor signaling pathways. Measurements of platelet aggregation and markers of platelet activation (GP IIb-IIIa and P-selectin detection by specific antibodies) show that clopidogrel resistance is detected in 31% of the patients on day 5 and 15% of the patients on day 30 of the treatment regimen [40]. A prospective study of PCI patients with non-ST segment elevation MI showed that up to 25% of the patients were resistant to clopidogrel [41]. When the patients were stratified into quartiles based on resistance to ADP-induced platelet aggregation, the most resistant patients had a 40% adverse event rate during a 6-month follow-up period so they are obviously being denied adequate protection based on inhibition of P2Y₁₂. It has also been reported that the antiplatelet activity of clopidogrel is blocked in patients treated with a widely used cholesterol lowering medication (atorvastatin) which is undoubtedly linked to the metabolism requirement for efficacy [42]. A second limitation of these drugs stems from their irreversible mechanism of action, which inactivates the P2Y₁₂ receptor for the lifetime of the platelet. While this is not a particular problem with aspirin, which is also irreversible, this feature has led to limited use of clopidogrel before PCI in patients who are at increased risk of undergoing CABG procedures, as the risk for bleeding following clopidogrel treatment requires postponement of the procedure for 5–7 days, or transfusion of large numbers of platelets during the procedure.

Limitations of anticoagulants – i.v.

Each anticoagulant has evolved unique issues. Replacement of unfractionated heparin has had mixed success. Although the current ACC/AHA guidelines (2002) prefer enoxaparin over unfractionated heparin, recent data do not support this preference. In the SYNERGY trial, enoxaparin was found not to be superior to unfractionated heparin [29]. In A to Z, for patients on a GP IIb-IIIa antagonist (tirofiban) and aspirin, enoxaparin was found to be non-inferior to standard heparin [43]. LMW heparins also have a narrow safety window. For example, in unstable angina patients treated with enoxaparin, a 25% dose increase in therapeutic level (1.25 mg kg⁻¹ vs.

1 mg kg⁻¹) produces an unacceptable number of bleeding events [44]. Direct thrombin inhibitors such as angiomas have also been studied as replacements for unfractionated heparin. In the REPLACE 2 trial of PCI, angiomas and provisional GP IIb-IIIa inhibitor compared favorably to heparin plus GP IIb-IIIa [45]. The primary end point of the trial combined efficacy and safety parameters and the angiomas arm of the trial was statistically not inferior to the heparin arm. However, the benefit related to reduction of bleeding with the use of angiomas is questionable. In REPLACE 2, the control group was likely to have been over anticoagulated as the observed ACT values in the heparin arm were higher than the recommended ACT range (200–300 s) for use of heparin in conjunction with GP IIb-IIIa. As angiomas is substantially more expensive than standard heparin, economic considerations also contribute to its limited use in PCI patients.

Limitations of anticoagulants – oral

Warfarin is the only anticoagulant in chronic use. While the drug provides tremendous benefit to affected individuals, its anticoagulant response is influenced by a variety of factors such that >50% of patients are usually outside of the therapeutic range. Due to the large variability in the anticoagulant effect of warfarin and its narrow therapeutic index, a large unmet clinical need exists for an anticoagulant with predictable fixed-dose usage. The need for a warfarin substitute has led to numerous drug development projects that have focused on inhibitors of coagulation proteases that specifically inactivate the protease active site. Ximelagatran, an oral thrombin inhibitor, was the first to show that the strategy of direct coagulation protease inhibition does translate into effective anticoagulation and leads to antithrombotic activity in deep vein thrombosis and atrial fibrillation patients [46]. Two studies suggest that ximelagatran is at least as effective as warfarin in preventing stroke in high-risk patients with atrial fibrillation [47]. The studies also showed that there are incidences of increase in liver enzymes which would require surveillance for potential liver toxicity in future patients. Unfortunately, safety problems of ximelagatran related to serious liver toxicity has led the FDA to recommend against approval of this thrombin inhibitor. While several new experimental agents with the potential to be an effective and low variability anticoagulant have been evaluated in clinical trials, none of these are available for therapeutic use, so the search for a warfarin replacement remains a work in progress.

PAR-1

While drugs that inhibit thrombin or prevent its formation are a mainstay in the armamentarium used for the treatment of arterial thrombosis, as of this writing, no efficacy trials have been performed to determine whether antagonists of PAR-1, the thrombin receptor on platelets, could provide a therapeutic benefit. Potent and selective PAR-1 antagonists capable of inhibition of thrombin-induced platelet aggregation have been

reported in the literature. Peptide mimetic antagonists such as RWJ-58259 are effective in models of thrombosis and vascular injury and could have potential as therapies for treating thrombosis and restenosis [48]. An oral PAR-1 antagonist, E-5555, is being developed as a drug candidate for ACS but definitive clinical efficacy trials have not been reported [49].

Future directions for antithrombotic drug development

New antithrombotics are required not only to overcome the limitations of the current drugs to better manage arterial ischemia, but also to address the inflammatory activities of platelets which contribute to progression of atherosclerotic disease. The successes and limitations of current therapies coupled with the advances made in our understanding of platelet biology are instructive in the design of new drugs to more effectively regulate validated targets, in the identification of new targets that may safely provide increased benefit and in the development of the proper combination of antithrombotics for the various arterial ischemic indications.

Agonist receptors

While platelets are activated by numerous agonists acting on multiple receptors, the only validated agonist receptor for drug discovery is P2Y₁₂. The requirements for improvements over clopidogrel are clear – more potent inhibition of P2Y₁₂; less variability of inhibition between different patients; no requirement for metabolism resulting in less delay in onset to action; and quicker recovery of platelet function following discontinuation of use. While these requirements can most likely be best achieved by an orally available reversible P2Y₁₂ antagonist, preclinical data indicate three promising candidates in development with different properties. One is cangrelor (AR-C69931MX), a nucleotide, intravenous compound that reversibly antagonizes P2Y₁₂ [50]. AZD-6140, an orally available direct-acting P2Y₁₂ antagonist [51], is presently being evaluated in phase II clinical trials. Prasugrel (CS-747), a thienopyridine prodrug similar to clopidogrel which is more rapidly converted to the active metabolite than is clopidogrel, has completed phase II trials and will be evaluated in phase III trials in ACS patients [52].

ADP also acts on P2Y₁, a G_q coupled receptor. Studies using either selective antagonists of P2Y₁ or P2Y₁₂, as well as gene-targeting strategies [53–56] have demonstrated distinct roles for these two ADP receptors. P2Y₁ is responsible for initiation of aggregation to ADP [55], while P2Y₁₂ is critical for amplification of the aggregation response by released ADP, and for stabilization of platelet aggregates and the growing thrombus [57]. In addition to the different roles of these two receptors in initiation and stabilization of thrombus growth, one could argue that the selective tissue distribution of P2Y₁₂ (platelets, megakaryocytes and glial cells), vs. P2Y₁ (which is ubiquitously expressed) makes it the preferred drug target. Although there are selective P2Y₁ antagonists which have been used as *in vitro* tools [58,59], none of these have been clinically evaluated as yet,

and may not have suitable pharmacokinetic properties to be viable drug candidates.

Secondary aggregation receptors

While the initial interaction of platelets during thrombosis is dependent upon GP IIb-IIIa, it has become apparent that signaling reactions initiated by platelet-platelet contact are required for thrombus stability. Several mediators of aggregation-induced signals have been identified. One is GP IIb-IIIa itself which becomes tyrosine phosphorylated and also associates with numerous signaling and cytoskeletal proteins following platelet aggregation. The importance of the 'outside-in' signaling in the enhancement of platelet aggregation was demonstrated by the generation of knock-in mice where tyrosine residues Y747 and Y759 were mutated to phenylalanine [60]. The so-called DiYF mice displayed selective impairment of outside-in signaling resulting in the formation of unstable aggregates. In addition, as shown in Fig. 2, *ex vivo* perfusion chamber experiments on type III collagen have shown that the DiYF mouse has defective thrombosis. Another protein involved in secondary platelet aggregation is CD40L, a tumor necrosis factor family member mainly expressed on activated T cells and platelets [see 61]. CD40L is cryptic in unstimulated platelets, but rapidly becomes exposed on the platelet surface after stimulation where it is subsequently cleaved, producing a soluble hydrolytic product termed sCD40L [61]. We have shown that mice lacking CD40L have a thrombosis phenotype and that normal thrombosis is regained upon infusion of sCD40L [62]. Interestingly, sCD40L, in addition to being a ligand for CD40, is also a ligand for GP IIb-IIIa, a reaction that depends upon its KGD sequence, a known GP IIb-IIIa binding motif. sCD40L also triggers outside-in signaling by tyrosine phosphorylation of GP IIIa, a reaction which is defective in the platelets from the DiYF mouse [63]. While inhibition of primary platelet aggregation and this secondary aggregation mechanism are both inhibited by GP IIb-IIIa antagonists, a potential drug target is the metalloproteinase responsible for CD40L cleavage. Another protein released upon platelet activation that functions to consolidate platelet thrombi is Gas6. Gene targeting of Gas6 also demonstrates a thrombosis phenotype [64]. Gas6 binds to three receptors on platelets, Tyro3, Axl and Mer, but genetic targeting of any one unexpectedly inhibits Gas6-induced platelet stimulation. However, as Gas6 also induces tyrosine phosphorylation of GP IIIa, apparently by a mechanism independent of binding to the integrin, it has been proposed that Gas-6 signaling could be therapeutically regulated through inhibition of Gas6-GP IIb-IIIa cross-talk [65].

Platelet-platelet contacts induce the activation of additional signaling mechanisms which are involved in aggregate stability. One involves Eph kinases and ephrins, specifically EphA4 and ephrinB1, which through receptor ligand interactions on the platelet surface enhance the binding of GP IIb-IIIa to immobilized fibrinogen in the presence of physiological agonists [66]. Recent work from our laboratory using both

oligonucleotide-based microarray analyses and mass spectrometric proteomics techniques has identified two additional receptor families that are involved. One involves two members of the SLAM family of adhesion receptors, SLAM and CD84; the other involves a novel protein termed PEAR1 (N. Nanda, M. Hart and D.R. Phillips, unpublished data). All proteins are exposed on the surfaces of unstimulated platelets and signal secondary to GP IIb-IIIa-mediated platelet-platelet contacts by becoming tyrosine phosphorylated. Therapeutic targeting of one or more of these secondary aggregation receptor systems in platelets is an attractive possibility as they appear to have a greater effect on thrombosis than they do on hemostasis.

Adhesion receptors

Several platelet adhesion receptors have been identified but we will focus on GPVI and GPIb α , the adhesion receptors that are not only involved in the adhesion of platelets to the highly thrombogenic fibrillar collagens in the vessel wall exposed following vascular injury but also contribute to platelet activation. Both receptors are attractive drug discovery targets as both are platelet specific. Under the high shear rates encountered in coronary and carotid arteries, the binding of VWF to the collagen surface triggers a transient interaction with GPIb α that allows for a more stable interaction of the platelet with the collagen surface via at least two collagen receptors, integrin $\alpha_2\beta_1$ and GPVI. Recent findings indicate that GPIb α and $\alpha_2\beta_1$ preferentially contribute to the adhesion process whereas engagement of GPVI triggers signaling events leading to platelet activation [67]. GPVI is non-covalently associated with the Fc receptor γ -chain and signals through the platelet via stimulation of multiple non-receptor tyrosine kinases. Interestingly, part of this signaling pathway may be common to GPIb α activation, and signals coming from these two receptors contribute to the activation of the receptor function of GP IIb-IIIa and platelet aggregation. Modulation of the GPVI receptor function is becoming an attractive target as platelets from GPVI-deficient animals, human platelets expressing low levels of GPVI, or platelets treated with a GPVI antibody, while unable to support thrombus growth [67–69], are nonetheless able to adhere on the collagen surface (via $\alpha_2\beta_1$ integrin and potentially another collagen receptor [70]) minimizing the effects on hemostasis [71,72]. GPIb α is a high shear rate-dependent thrombosis receptor that affects recruitment of platelets at sites of vascular injury (on the collagen present in the subendothelium and on adhering platelets) with minor impact on venous thrombotic process. Modulation of the VWF/GPIb α axis has been the subject of many investigations with promising animal experimental results, but severe thrombocytopenia has been associated with the use of antibodies against GPIb α , thus reducing the general interest of the scientific community for several years. Nevertheless, novel strategies targeting the VWF/GPIb α axis through snake venom proteins cleaving GPIb α , VWF peptides or antibodies against VWF are reviving this strategy.

Signaling pathways

The extensive repertoire of platelet functions, while initiated by receptors, is regulated by signal transduction pathways. While these pathways have not been known as drug discovery targets, two observations suggest that they are worthy of consideration. First, the remarkable success of Gleevec, an Abelson tyrosine kinase inhibitor, has proven efficacy in the treatment of chronic myelogenous leukemia and other cancers. While the multiple functions of this kinase in diverse cell types predicted toxicity, clinical data have shown that the benefits far outweigh the liabilities. The success of this drug suggests that signal transduction pathways, though redundant for multiple signaling systems in diverse cell types, are worthy of consideration as therapeutic drug discovery targets. This conclusion is supported by the analysis of numerous gene-targeted mouse strains which have led to the surprising conclusion that the phenotype achieved by the disruption of any specific gene is often limited, even for genes involved in signal transduction. A second observation is that the platelet stimuli often induce diverse responses. For example, any one of the primary platelet agonists are capable of producing a spectrum of responses which could have pathological implications, e.g. aggregation caused by the activation of the receptor function of GP IIb-IIIa, expression of procoagulant activity of prothrombinase or FXase to catalyze the production of thrombin, generation of vasoactive substances such as TXA₂ and serotonin to induce vasoconstriction, release of proinflammatory proteins like sCD40L, RANTES and TGF- β to affect vascular inflammation including the progression of atherosclerosis, the release of growth factors such as PDGF to affect vascular remodeling, and the activation of secondary aggregation receptors such as SLAM, CD84 and the ephrins to stabilize thrombi and cause vascular occlusions. As many of these responses would be expected to be regulated by a specific pathway, it is reasonable to expect that these responses could be individually regulated. If true, this approach could inhibit platelet-dependent pathologies without compromising primary hemostasis. One potential example is PI-3 kinase and the regulation of the adhesive function of GP IIb-IIIa [73].

The roles of secondary signaling events downstream of platelet surface receptors have been elucidated through gene-targeting studies in mice, and subsequent evaluation of their platelet phenotypes using both *in vitro* and *in vivo* techniques. As certain key platelet agonists such as ADP, thrombin, and TXA₂ all activate platelets through G protein-coupled receptors, genetic targeting of individual α -subunits of G proteins has been a successful strategy in studying platelet signaling downstream of receptor activation. Characterization of platelets from mice lacking G_q, G₁₂ and G₁₃, identified these three proteins as key mediators for ADP and TXA₂ receptors [27,74–76]. Targeting of additional subunits (G₂, G₁₃, and G₁₂) showed little or no effect on platelet phenotype, which could be due to lack of coupling of these subunits to critical platelet receptors, or due to redundancy in the signaling pathways. Thrombin signaling has been shown to be affected by lack of G_i, G_q, and

G₁₃, to varying extents. In mice lacking both G_q and G₁₃, no platelet activation was possible by ADP, TXA₂, or thrombin [27], suggesting that at least G_q or G₁₃ is required to induce some activation, and that activation of G_i-type proteins alone is not sufficient for activation of mouse platelets. In these G_q/G₁₃ double-deficient platelets, adhesion of platelets to collagen was not affected; however, aggregation in response to collagen fibrils as well as formation of stable aggregates on collagen-coated surfaces was completely eliminated. In addition to targeting of G protein subunits, targeting molecules involved in kinase signaling pathways have resulted in mice with impaired platelet functions, which is not unexpected given the fact that kinases of the src family (Csk, src) have been shown to be physically associated with the cytoplasmic domain of GP IIb-IIIa [77]. The platelet phenotype of mice lacking the tyrosine kinase syk, critical for downstream signaling through the collagen receptor GPVI, and also activated during outside-in signaling and activation of GP IIb-IIIa, exhibited defects in platelet activation induced by ADP ± epinephrine [78]. Mice lacking the adapter protein SLP-76, which is on the syk signaling pathway, have been shown to have defects in GP IIb-IIIa signaling and collagen receptor responses [79]. The important role of downstream signaling molecules identified through gene-targeting studies may provide new opportunities for therapeutic intervention for blockade of platelet activation, thrombus formation, and adhesion.

Evolving paradigm on the relationship of thrombosis to inflammation

Not only are platelets critical players in mediating thrombosis, but recently their role in inflammation has become more appreciated. Although it is widely accepted that inflammatory activities that orchestrate the progression of atherosclerosis are derived from 'traditional' inflammatory cells such as monocytes and neutrophils, emerging data suggest that products released from platelets during thrombosis are actively involved in this process and that platelets are a primary source of inflammatory proteins within the circulation. For example, Schober et al. [80] reported that platelets deposit RANTES onto endothelial cells in the injured vessel wall, and that this interaction is mediated by P-selectin, a surface receptor mediating the attachment of platelets to leukocytes and endothelium. Additional work from this laboratory showed that infusion of activated platelets into the apoE^{-/-} mouse greatly enhanced the rate of atherosclerotic lesion progression [81]. The exposure of P-selectin following platelet activation is a key mediator of platelet-leukocyte interaction, and facilitates atherosclerotic lesion development, as demonstrated by Burger and Wagner [82]. In patients with acute MI, platelet-leukocyte interaction is increased compared with controls, and P-selectin levels have been shown to remain increased for at least a month following initial presentation in ACS patients with non-ST segment elevation [83], and even in patients with stable coronary artery disease. Antiplatelet therapy with a P2Y₁₂ antagonist plus aspirin was shown to decrease platelet-monocyte interactions that occur after

coronary stenting [84], an effect not observed with anticoagulants plus aspirin, suggesting that P2Y₁₂ antagonism had an anti-inflammatory effect distinct from its antithrombotic mode of action. Targeting of CD40L, another platelet-derived inflammatory protein, either through a blocking antibody [85] or via gene-targeting [86], greatly inhibited lesion progression in either LDLR^{-/-} or apoE^{-/-} mice, respectively. Soluble CD40L (sCD40L), the shed hydrolytic product of CD40L, 95% of which is platelet-derived, has been shown to be a primary risk factor for atherosclerosis/thrombosis [87]. In addition, the binding of platelet-derived sCD40L to endothelial cells can lead to the expression of tissue factor, a potent procoagulant. Clopidogrel has been shown to inhibit ADP-induced CD40L expression, and to lower CD40L levels in patients undergoing PCI [88], while aspirin does not. Thus, some forms of antiplatelet therapy, including P2Y₁₂ inhibition, can inhibit platelet pro-inflammatory responses. Finally, recent data show that targeting of GP Ib-IX-V complex, a platelet adhesion receptor, in the apoE^{-/-} mouse blocks leukocyte recruitment and the development of atherosclerotic lesions [89]. Thus, studies of the role of platelets in inflammation may provide new potential targets for CVD through inhibition of atherosclerosis and/or thrombosis.

Platelet monitoring

The pharmaceutical industry and drug approval agencies expect that the early inroads into personalized medicine in the administration of selected chemotherapies will ultimately extend to all drug classes. These early examples include screening for HER2 positive individuals in the treatment of breast cancer with Herceptin and the screening for EGF receptor for the treatment of lung cancer with gefitinib [90]. More generally, by the time this manuscript is published, the CYP450 screen will most likely be available to detect the various isoforms of P450 which will be useful in projecting drug levels in individuals treated with a wide variety of drugs. While such strategies will be of value in selecting and dosing antithrombotics, particularly for chronic use, access to blood of patients treated with antithrombotics continues to provide the best opportunity for monitoring the effect of any therapy or any combination of therapies on each patient being treated. While suitable assays are available to monitor anticoagulants, the monitoring of antiplatelet drugs is not routinely performed.

Evaluations of data from the current antithrombotics and their limitations have defined the assays required to bring personalized medicine to the patient treated with antiplatelet drugs to prevent arterial thrombosis. First, platelet function should be monitored in the context of thrombosis. Platelet thrombosis *in vivo* is initiated by adhesive proteins exposed on the vessel wall and stable thrombi result following adhesion, activation, aggregation, and thrombus stabilization, all occurring under conditions of shear. As thrombus stability is one of the issues, continuous monitoring of thrombus formation is essential for determining the effects of drugs that effect targets

involved in thrombus stability, for example, in prostanoid metabolism and in ADP release. Although methods currently available such as light transmittance aggregometry, the Ultegra Rapid Platelet Function Assay and platelet activation markers such as P-selectin expression are effective in monitoring the ability of end products of any one of these pathways to activate platelets, they are ineffective in monitoring thrombosis, the physiological response of platelets to thrombogenic surfaces under shear. Furthermore, while the PFA-100 device is capable of monitoring the time required for a platelet plug to form in apertures coated with ADP or collagen, it does not provide a continuous monitoring of the thrombotic process. A second requirement for the personalized monitoring of drugs to prevent arterial thrombosis is that it should not only be responsive to diverse drug classes but also be capable of determining the net effect on thrombosis achieved by combinations of antithrombotic therapies. At the present time, the four drug classes used to treat patients at risk for arterial thrombosis, aspirin, GP IIb-IIIa antagonists, clopidogrel and anticoagulants, are used in combinations not properly evaluated for their net effect on thrombosis. Drugs against additional platelet and coagulation protein targets will become available. Required are technologies that will readily permit evaluation of how combinations of these drugs affect thrombosis in patients receiving these drugs. A third requirement is that the method must be capable of monitoring individual differences in response to therapy. As outlined above, individual differences in response to aspirin and clopidogrel have been observed – differences which appear to affect clinical outcome. While currently available techniques such as light transmittance aggregometry, Ultegra, or PFA-100 are useful in monitoring responsiveness to both of these drugs as monotherapy, they are ill-suited to measure individual differences when combinations of drugs are employed, the emerging norm. It is also anticipated that this problem would be amplified when drugs against additional targets would be introduced. Finally, as the anticoagulants routinely used in blood collection such as citrate or direct thrombin inhibitors such as PPACK affect thrombosis, the monitoring method must be capable of assaying non-anticoagulated samples of blood. Perhaps the best example of anticoagulant interference in antithrombotic monitoring is in the development of GP IIb-IIIa antagonists where citrate anticoagulation markedly overestimates antiaggregatory activity [91]. The optimal method for monitoring individual thrombotic potential must be capable of either performing the assay in the absence of anticoagulants or of being able to determine how any given anticoagulant affects the assay.

Use of perfusion chamber technology has perhaps provided the best hope of measurement of the thrombotic potential of individuals being treated with combinations of antithrombotic drugs. Perfusion chambers were designed 30 years ago in order to characterize the thrombotic process under shear conditions. The different types of perfusion chambers described in the literature can be classified according to their geometry (circular, annular, flat chambers) or the surfaces (blood vessels, isolated proteins) exposed to flowing blood. These techniques confer the

advantage of studying platelet interactions with a thrombogenic surface under specific conditions of shear rate with either non-anticoagulated or anticoagulated blood. Major contributions to the field of thrombosis have originated from use of perfusion chambers. For example, the critical role of VWF and its interactions with GP Ib and GP IIb-IIIa to mediate platelet adhesion and thrombus growth under arterial shear rates, the involvement of GP VI and of the integrin $\alpha\beta 1$ in mediating platelet adhesion and activation on collagen. Inhibitors of P2Y₁₂ and Cox-1 have also demonstrated antithrombotic activities in this system [92]. However, perfusion chambers are mostly utilized by academic institutions or by pharmaceutical and biotechnology companies in order to identify or validate targets and to develop antithrombotic drugs. Several limiting factors have prevented their use as a bedside device for monitoring drug efficacy in clinical trials – the skill required to determine thrombus size was not readily available in clinical settings; quick readouts for the patient were not available; end point quantifications left investigators without knowledge of the kinetics of thrombus formation, the more critical information.

Results from several laboratories, however, have made progress in modifying these devices to circumvent these difficulties. Figures 3 and 4 illustrate the utility of monitoring the kinetics of thrombosis in perfusion chamber assays. In one instance, using non-anticoagulated samples of blood, we have shown that inhibition of P2Y₁₂, Cox-1, or FXa did not significantly reduce thrombus size after a 4-min perfusion period over a collagen-coated surface. However, when more than one of these targets was inhibited, pronounced antithrombotic activity was observed. In another experiment, when human blood was anticoagulated with an FXa inhibitor and perfused through a chamber in the real-time assay, we observed that P2Y₁₂ antagonism with clopidogrel did not affect the

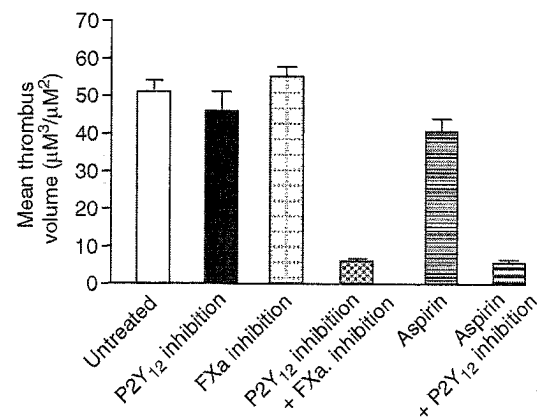


Fig. 3. Synergism between P2Y₁₂ antagonism, Factor Xa inhibition and aspirin. As indicated unanticoagulated blood was treated with an inhibitor for P2Y₁₂ (100 µM 2MeSAMP) or Factor Xa (10 µM C921-78). Aspirin-treated was from aspirin-treated individuals. The treated blood was perfused through a chamber coated with type III collagen at 1000 s⁻¹ for 4 min and quantified as described [23].

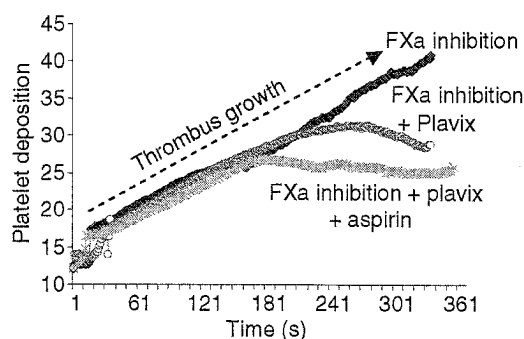


Fig. 4. Synergism between aspirin and P2Y₁₂ inhibition in blocking thrombus growth. Blood from a control individual, an individual treated with clopidogrel, or an individual treated with aspirin and clopidogrel was anticoagulated with a Factor Xa inhibitor, treated with rhodamine 6G to label platelets, and then perfused through a chamber coated with type III collagen at 1000 s⁻¹. The continuous accumulation of fluorescence was used to quantify platelet thrombus formation.

initial thrombus growth triggered by fibrillar type III collagen under arterial shear rates. However, clopidogrel caused the thrombi formed during the first 3 min of perfusion to dissociate. Control thrombi formed in the absence of clopidogrel were stable and continued to grow. This demonstrates the limitations of end point analysis as the measured anti-thrombotic activity is dependent on the time of analysis. Retrospectively, this explains an apparent discrepancy found in the evaluation of the phenotype of P2Y₁₂^{-/-} mice [57]. P2Y₁₂^{-/-} mice demonstrate a cyclic thrombotic process *in vivo*, but only a qualitative difference (i.e. more loosely packed thrombi) was observed after perfusion of non-anticoagulated blood for 2.5 min over type III collagen. Evaluation of mono- and combination therapies in this assay confirmed the antithrombotic efficacy of the different anti-platelet therapies, with GP IIb-IIIa antagonists being inhibitors of thrombus growth, aspirin and P2Y₁₂ antagonist destabilization agents, the combination aspirin + P2Y₁₂ antagonism showing a faster destabilization activity (Fig. 4). Thus, perfusion chamber technology is suited to meet the requirements of personalized medicine for individuals receiving antithrombotic therapies as the measurement is on thrombosis, it is responsive to diverse drug classes and it can be performed in the absence of anticoagulants. Future discoveries are required to adapt such technologies to devices that are readily available to individual patients. Finally, several laboratories have reported measurements of the inflammatory activities of platelets, e.g. sCD40L plasma levels, P-selectin expression, formation of platelet-leukocyte complexes. As recent data show that the inflammatory activity of platelets is important in the progression of atherosclerosis, it would also be desirable to develop methods to rapidly quantitate the platelet inflammatory activity in patients.

Application of this personalized medicine approach to antithrombotic therapies does have significant hurdles to overcome before it can be used to reliably modify therapy.

First, a bedside monitor of the thrombotic potential of individual patients needs to be developed. Second, recognizing that individuals will undoubtedly be heterogeneous with respect to vessel wall thrombogenicity, including the local shear environment, results using this device need to be correlated with clinical outcomes.

Conclusions

The current repertoire of drugs for the treatment of patients at risk for arterial thrombosis (e.g. ACS, diabetes, poststroke, peripheral artery disease, post-AMI) currently includes four classes of drugs – aspirin, GP IIb-IIIa antagonists, thienopyridines, and anticoagulants. Although each of these drug classes has proven efficacies for different indications, each has limitations that continue to permit thrombotic events during their use. In addition, emerging data suggest that a significant percentage of individuals treated with aspirin or clopidogrel do not receive the expected therapeutic benefit from therapy because of a decreased responsiveness by their platelets. Future directions in addressing these limitations will proceed in two parallel directions. On the one hand, it can be anticipated that new drugs, either offering improvements against known, validated targets, or against recently identified targets, will be forthcoming. Recognizing that platelets are now known to be directly involved in vascular inflammation including that which leads to the progression of atherosclerotic disease, it can be anticipated that some of these new therapeutic strategies will not only better address arterial thrombosis, but also inhibit the ability of platelets to deliver inflammatory proteins and growth factors which affect atherosclerotic lesion development. On the other hand, it has now become apparent that improvements are required in the devices used to monitor the thrombotic potential of individuals receiving therapy, both for the development of new antithrombotic drugs and to measure the effectiveness of combined antithrombotic therapies. It would appear that the most effective device is that which measures thrombosis in real time, is accessible to the patient at the point of drug administration, and can be performed in the absence of anticoagulation. Such a device would be capable of monitoring the activities of new classes of antithrombotics, of measuring variances of individual responses, and in evaluating the effectiveness of combined antithrombotic therapies.

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